REMARKS

The amendments made herein are believed to place the claims in condition for allowance, and do not require additional search and consideration of the claims. Therefore, it is requested that, despite the fact that the Office Action mailed October 19, 2010 has been made final, the Examiner enter and consider the amendment/arguments herein.

The claims

Claim 11 has been amended to address concerns of the Examiner and to clarify the claim. Support for "pharmaceutically acceptable" salt is found throughout the specification, *e.g.* on page 23, lines 10-22. Claims 1, 7-10 and 12-17 are withdrawn from consideration. Claim 4, which was improperly withdrawn from consideration, has been reinstated, per the discussion on page 2 of the October 19, 2010 Office Action, and designated as an "original" claim. New claims 18 and 19 are supported throughout the specification, *e.g.* at page 25, lines 6-7. Claims 2-6, 11, 18 and 19 are under examination.

Written description rejection of claims 2-6 and 11

Applicants disagree with the Examiner's allegation that the claims directed to "a salt [of clavulanic acid] or an active ester form thereof that are hydrolyzed *in vivo* to clavulanic acid", in an amount effective to achieve the recited functions, lack adequate written description, for reasons of record. Nevertheless, in an effort to expedite prosecution, claim 11 is amended to delete the recitation of "activated ester forms [of clavulanic acid] that are hydrolyzed *in vivo* to clavulanic acid." Claim 11 now recites "clavulanic acid or a pharmaceutically acceptable salt thereof." It is noted that salts of clavulanic acid need not be hydrolyzed to clavulanic acid. Therefore, a skilled worker, upon reading the description of the specification of adequate dosages of clavulanic acid, would have in his or her possession a description of the dosages of salts of clavulanic acid. Support for suitable amounts of clavulanic acid is found throughout the specification, *e.g.*, on page 5, lines 4-5, page 7, lines 24-25, page 24, lines 25-27, and pages 30-33, 35, 37, 40, 42 and 44, as outlined in the Reply filed on August 10, 2010.

Furthermore, the application discloses administering a potassium salt of clavulanic acid during *in vivo* testing. See, *e.g.*, page 32, lines 17-18.

It is requested that the rejection be withdrawn.

Obviousness rejection

The Examiner has rejected claims 2-6 and 11 as allegedly being obvious in view of Tew *et al.* (WO 97/10247) ("Tew *et al.* "), in view of several secondary references.

The Examiner alleges that Tew *et al.* discloses compounds, which are ester derivatives (forms) [emphasis added] of clavulanic acid, that "function as inhibitors of lipoprotein associated phospholipase A_2 (Lp-PLA_2) and are, therefore, useful in a method for treating a disease state associated with the activity of the enzyme Lp-PLA_2", and that among such disease states is Alzheimer's disease.

Applicants contend that the claims which were pending when the August 10, 2010 Response was filed were not rendered obvious by the cited references, for reasons of record. Nevertheless, in an effort to expedite prosecution, claim 11 has been amended to remove the recitation of "activated ester forms [of clavulanic acid] that are hydrolyzed *in vivo* to clavulanic acid." The amended claim is directed to, *inter alia*, the use of clavulanic acid or a pharmaceutically acceptable salt thereof to treat a cognitive disorder (such as, *e.g.*, Alzheimer's disease) in a patient in need of said treatment.

Tew *et al.* does not teach using clavulanic acid or a pharmaceutically acceptable salt thereof to treat a patient in need of treatment for a cognitive disorder, as required by the amended claims. In fact, Tew *et al.* does not even mention or suggest clavulanic acid and pharmaceutically acceptable salts thereof, and therefore does not suggest using clavulanic acid or its pharmaceutically acceptable salts to treat a patient in need of treatment for a cognitive disorder, such as Alzheimer's disease. The reference relates only to derivatives of clavulanic acid (in particular certain defined amide and esters of clavulanic acid) that can inhibit lipoprotein associated phospholipase A2 (LP-PLA_2).

According to Tew *et al.*, LP-PLA_2 is an enzyme that converts phosphatidylcholine to lysophosphatidylcholine, which is a substrate that contributes to the accumulation of cholesterol and the development of atherosclerosis. Based on this relationship between LP-PLA_2 and atherosclerosis, Tew *et al.* suggests using derivatives of clavulanic acid to

treat atherosclerosis. However, as was noted in the Reponse filed on August 10, 2010, Tew *et al.* does not disclose any relationship between LP-PLA_2 and Alzheimer's disease. Tew *et al.* does not identify any scientific data linking LP-PLA_2 with Alzheimer's disease, and it does not provide any experimental data supporting such a proposition. At best, Tew *et al.* discloses the mere germ of an idea that derivatives of clavulanic acid may be used in treating Alzheimer's disease. (*See*, for example, [page 4, lines 8-14], "In addition, compounds of formula (I) may have a general application in any disorder that involves lipid peroxidation in conjunction with enzyme activity, for example in addition to conditions such as atherosclerosis and diabetes, other conditions such as rheumatoid arthritis, stroke, inflammatory conditions of the brain such as Alzheimer's Disease, myocardiai infarction, reperfusion injury, sepsis, and acute and chronic inflammation. Further such conditions include various neuropsychiatric disorders such as schizophrenia. Further applications include any disorder that involves activated monocytes, macrophages or lymphocytes, as all of these cell types express Lp-PLA_2. Examples of such disorders include psoriasis.") Without a firm understanding of the role that Lp-PLA_2 might play in mechanisms of cognitive disorders such as dementia, which are complex, poorly understood conditions, a skilled worker could not have predicted with any degree of confidence that any inhibitor of Lp-PLA_2, let alone clavulanic acid, could be used to treat such conditions.

A review of the scientific literature indicates that no connection between Lp-PLA_2 and dementia, including Alzheimer's disease, had been demonstrated until long after the effective filing date of the present application (August 16, 1999) or the date of publication of Tew *et al.* (March 20, 1997). Clearly, although Tew *et al.* may have postulated theoretically that derivatives of clavulanic acid might be used to treat Alzheimer's disease, there was no sound scientific basis for such a hypothesis, or evidence that this was the case.

Several representative publications supporting this conclusion are discussed below and are attached for the convenience of the Examiner:

1. van Oijen *et al.* (2006) *Annals of Neurology* 59, 139-144 states in its Abstract that "this is the first study (emphasis added) to our knowledge that shows LP-PLA_2 is associated with the risk of dementia independent of cardiovascular and inflammatory

factors and provides evidence for a potential role of LP-PLA_2 in identifying subjects at risk of dementia." This paper was published in 2006, seven years after the effective filing date of the present application and nine years after the publication of Tew *et al.* To explain the basis of their hypothesis of a connection between LP-PLA_2 expression and the risk of dementia, the authors of van Oijen *et al.* refer to their own findings in 2005 that higher levels of LP-PLA_2 were associated with coronary heart disease and with stroke, and to other papers published after the filing date of the present application which suggested that inflammatory markers, cardiovascular risk factors and cerebrovascular disease had been suggested to play a role in dementia. These papers were all published well after Tew *et al.*was published. Furthermore, the authors caution on page 143, column 1, first full paragraph, that the signficance of the correlation they observed was uncertain: "Note that there was not a compelling gradient of risk of dementia for levels of LP-PLA_2." They also point out on page 143, column 2, second paragraph that one of their rationales for looking for this connection (and the rationale set forth in Tew *et al.*) - the accumulation of cholesterol because of the action of LP-PLA_2 - may not even be an important factor in dementia: "The role of cholesterol in dementia is actually far less clear than its role in cardiovascular disease. Moreover, in our data, cholesterol was not associated with the risk of dementia."

2. Chalbot *et al.* (2009) *Clin. Chem* 55, 2171-2179 reports that "In this study, we have shown for the first time (emphasis added) a significant increase of sPLA2 [a form of phospholipase 2] activity in AD [Alzheimer's disease] compared with age-matched controls" (page 7, last sentence). This paper was published in 2009. Not only was this study published long after the effective filing date of the present application, but the form of phospholipase A2 was quite different from that discussed in Tew *et al.* The phospholipase A2 of Chalbot *et al.* is a *secreted* (extracellular) form of of the protein, referred to as sPLA2, which was found in cerebral spinal fluid (CSF), and which is Ca^{+2}-dependent. This differs from the LP-PLA_2 (also known as platelet-activating factor acetylhydrolase (PAF-AH)) discussed in Tew *et al.*, at least because LP-PLA_2 is Ca^{+2}-independent, and because LP-PLA_2 is considerably larger (about 26-45 kDa) than the secreted sPLA2 form (about 14-19 kDa).These comparisons are summarized in the first paragraph on page 2 of the publication. Furthermore, the authors of Chalbot *et al.* caution

that, even as late as 2009, "despite several studies, the functions of brain PLA2s are either unknown, diverse or controversial" (page 2, end of second full paragraph).

3. Koshy *et al.* (2010) *Journal of Alzheimer's Disease* 21, 775-780 report that, in spite of suggestions that high Lp-PLA_2 activity is a risk factor for dementia, members of a Japanese population that carry a loss of function polymorphism in the Lp-PLA_2 gene, and thus exhibit low levels of activity of Lp-PLA_2, do not exhibit a reduced risk of AD. This finding was published as recently as 2010.

These publications show that any correlation between Lp-PLA_2 activity and cognitive disorders, including dementia, such as in Alzheimer's disease, was not demonstrated until many years after the hypothetical statement of such a correlation had been made by Tew *et al.*; and that even the authors of papers which reported such a correlation cautioned that the relevance, if any, of the correlation was not well understood.

Even if a correlation had been demonstated between levels of Lp-PLA_2 activity and dementia, such as in Alzheimer's disease, at the time of filing of the application or at the time of publication of Tew *et al.*, which is not admitted, there certainly was no evidence to indicate whether such an increase in Lp-PLA_2 activity was the cause of, or a consequence of, neurodegenerative processes, or whether activities of Lp-PLA_2 were primary or secondary. Therefore, a skilled worker could not have extrapolated from such a correlation that an inhibitor of Lp-PLA_2 activity would have had an effect on dementia (*e.g.* in Alzheimer's disease). There certainly was no evidence at that time that inhibitng Lp-PLA_2 activity would have any effect on dementia.

In fact, as was stated as late as April, 2008 in a review of Lp-PLA_2 and cancer, by the well-respected academic physician, Dr. Mark Levin, published as http://cancertreatments.typepad.com/cancer_treatment/2008/04/lipoprotein-ass.html, a copy of which is attached to this Reply for the convenience of the Examiner, "The connection to cardiovascular disease and Alzheimer's remains unproven; neither are the effects of interventions to reduce the level of this marker known. Empire considers this test to be not medically necessary."

The secondary references cited by the Examiner do not remedy the defects of Tew *et al.*

Accordingly, for at least the above reasons, Tew *et al.*, by itself or in combination with the cited secondary references, does not render amended claims 2-6 and 11 obvious. Withdrawal of the rejection is requested.

In view of the preceding amendments and arguments, it is believed that the application is in condition for allowance, which action is respectfully requested.

Should any additional fee be deemed due, please charge such fee to our Deposit Account No. 22-0261, referencing docket number 41890-290023 and advise us accordingly.

Respectfully submitted,

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Cerebrospinal Fluid Secretory Ca^{2+}-Dependent Phospholipase A_2 Activity Is Increased in Alzheimer Disease

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Abstract

BACKGROUND: The phospholipase A_2 (PLA2) family comprises multiple isoenzymes that vary in their physicochemical properties, cellular localizations, calcium sensitivities, and substrate specificities. Despite these differences, PLA2s share the ability to catalyze the synthesis of the precursors of the proinflammatory mediators. To investigate the potential of PLA2 as a biomarker in screening neuroinflammatory disorders in both clinical and research settings, we developed a PLA2 assay and determined the predominant types of PLA2 activity in cerebrospinal fluid (CSF).

METHODS: We used liposomes composed of a fluorescent probe (bis-Bodipy® FL C11-PC [1,2-bis-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-undecanoyl)-*sn*-glycero-3-phosphocholine]) and 1,2-dioleoyl-L-α-phosphatidylcholine as a substrate to measure CSF PLA2 activity in a 96-well microtiter plate format. We established the type of CSF PLA2 activity using type-specific inhibitors of PLA2.

RESULTS: Using 5 μL CSF per assay, our PLA2 activity assay was reproducible with CVs <15% in 2 CSF samples and for recombinant secretory Ca^{2+}-dependent PLA2 (sPLA2) in concentrations ranging from 0.25 to 1 μmol/L. This PLA2 assay allowed identification of sPLA2 activity in lumbar CSF from healthy individuals 20–77 years old that did not depend on either sex or age. Additionally, CSF sPLA2 activity was found to be increased ($P = 0.0008$) in patients with Alzheimer disease.

CONCLUSIONS: Adult human CSF has sPLA2 activity that can be measured reliably with the assay described. This enzyme activity in the CSF is independent of both sex and age and might serve as a valuable biomarker of neuroinflammation, as we demonstrated in Alzheimer disease.

Nearly 22 different phospholipase A_2 (PLA2,[5] EC 3.1.1.4) enzymes have been identified and broadly classified into several families based on their structure, cellular localization,

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Authors' Disclosures of Potential Conflicts of Interest: No authors declared any potential conflicts of interest.

substrate specificity, and calcium requirement for catalytic activity: (1) the secretory (extracellular) Ca^{2+}-dependent PLA2 (sPLA2) family of 10 isoenzymes with low molecular weight (14–19 kDa) that requires mmol/L calcium concentrations for catalytic activity; (2) the cytosolic Ca^{2+}-dependent PLA2 (cPLA2) family of 3 isoenzymes with high molecular weight (61–114 kDa) that requires μmol/L calcium concentrations for binding to lipid substrates; (3) the intracellular Ca^{2+}-independent PLA2 (iPLA2) family of 2 isoenzymes with high molecular weight (84–88 kDa); (4) the platelet-activating factor acetylhydrolase (PAF-AH) family of 4 isoenzymes with low molecular weight (26–45 kDa) that are calcium independent; and (5) the lysosomal PLA2 family of a 45-kDa isoform that does not require calcium for catalytic activity (1).

Despite their differences, PLA2s share the ability to catalyze the hydrolysis of the *sn-2* ester bonds of glycerophospholipids, resulting in the production of free fatty acids (e.g., arachidonic acid and docosahexaenoic acid) and lysophospholipids. These metabolites have a variety of physiologic effects, e.g., regulation of gene expression and maintenance of neural membrane integrity, and serve as precursors for the synthesis of proinflammatory mediators such as eicosanoids (prostaglandins and leukotrienes) and platelet-activating factor (2). Although these PLA2-derived metabolites, at low concentrations, contribute to normal functions, their excessive production and accumulation can lead to pathological processes.

Second to adipose tissue, the brain has the highest lipid concentration. Dysregulation of lipid metabolism that results in an upregulation of PLA2 is of particular interest in the context of the central nervous system and has already been considered in a number of neurological, neurodegenerative, and psychiatric disorders including epilepsy, ischemia, Alzheimer disease (AD), Parkinson disease, schizophrenia, and mood disorder (2,3). But, despite several studies, the functions of brain PLA2s are either unknown, diverse, or controversial.

The availability of a simple, reproducible, and sensitive assay is needed to facilitate studies regarding the role of PLA2 isoenzymes in regulating physiological and pathological functions in the central nervous system. We report here the development and validation of such an assay for measurement of PLA2 activity in cerebrospinal fluid (CSF).

Materials and Methods

CHEMICALS

Recombinant mouse group X secretory PLA2 (4,5) was a kind gift from Tauseef R. Butt (LifeSensors Inc.). Bis-Bodipy® FL C_{11}-PC [1,2-bis-(4,4-difluoro-5,7-dimethyl-4-bora-3a, 4a-diaza-*s*-indacene-3-undecanoyl)-*sn*-glycero-3-phosphocholine] was purchased from Invitrogen; AACOCF3 (arachidonyl trifluoromethyl ketone), BEL (bromoenol lactone), and thioetheramide-PC (1-palmitylthio-2-palmitoylamido-1,2-dideoxy-*sn*-glycero-3-phosphorylcholine) were from Cayman Chemical.; PG (L-α-phosphatidylglycerol), DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine), and other chemical products were from Sigma Aldrich.

[5]Nonstandard abbreviations: PLA2, phospholipase A_2; sPLA2, secretory Ca^{2+}-dependent PLA2; cPLA2, cytosolic Ca^{2+}-dependent PLA2; iPLA2, intracellular Ca^{2+}-independent PLA2; PAF-AH, platelet-activating factor acetylhydrolase; AD, Alzheimer disease; CSF, cerebrospinal fluid; bis-Bodipy FL C_{11}-PC, 1,2-bis-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-undecanoyl)-*sn*-glycero-3-phosphocholine; AACOCF3, arachidonyl trifluoromethyl ketone; BEL, bromoenol lactone; thioetheramide-PC, 1-palmitylthio-2-palmitoylamido-1,2-dideoxy-*sn*-glycero-3-phosphorylcholine; PG, L-α-phosphatidylglycerol; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; NINCDS-ADRDA, National Institute of Neurological and Communicative Disorders and Stroke/ Alzheimer's Disease and Related Disorders Association; MMSE, Mini-Mental State Examination; FI, fluorescence intensity; NBD C6-HPC, 2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino)hexanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine; IL, interleukin; TNF-α, tumor necrosis factor-α; AA, arachidonic acid; PGE2, prostaglandin E_2.

STUDY PARTICIPANTS AND CSF SAMPLING

We generated the study population from retrospectively collected lumbar CSFs available for research purposes. The study was approved by the ethics committees of Eastern Norway and the University of Gothenburg and by the Institutional Review Board of the New York State Institute for Basic Research in Developmental Disabilities. Informed consent was obtained from all participants in accordance with the provisions of the Helsinki Declaration. Forty-two healthy individuals, without any cognitive disturbance, were actively recruited and selected to participate in this study, plus an additional group of 33 AD patients diagnosed with probable AD according to criteria established by the work group of the National Institute of Neurological and Communicative Disorders and Stroke/Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) (6). Mini-Mental State Examination (MMSE) scores were documented for most AD patients and for some controls (7). We determined the apolipoprotein E isoform by isoelectric focusing/immunoblotting using serum samples (8). The clinical features of the participants in this study are summarized in Tables 1 and 2. Venipuncture was performed to obtain serum, and CSF was obtained by lumbar puncture from the L3/L4 or L4/L5 intervertebral space; the first 12 mL CSF was collected, centrifuged at 2000*g* for 10 min at −4 °C, and aliquoted in 1-mL polypropylene tubes. All samples were sent in dry ice from Sahlgrenska University Hospital to New York State Institute for Basic Research and kept at −80 °C until used.

CONTINUOUS FLUORESCENT ASSAY FOR PLA2

We prepared fluorescence-labeled liposomes similarly to a described method (9). To optimize the conditions for the measurement of PLA2 activity in CSF, we adjusted parameters such as concentration and composition of phospholipids. Briefly, 388 μg DOPC and 97 nmol bis-Bodipy FL C_{11}-PC were mixed in 970 μL chloroform. After drying under vacuum, we added 970 μL sucrose/Tris buffer (50 mmol/L Tris-HCl, pH 7.4, 250 mmol/L sucrose, 0.02% sodium azide), followed by thorough and repeated mixing with a Vortex-mix over 5 min at room temperature. The suspension was then ultrasonicated for 10 min on ice using an ultrasonic cell disrupter (Branson Digital Sonifier) at 50 W sonic energy. The liposomes were stored at −20 °C until used. Liposomes containing other proportions and/or types of phospholipids were prepared by comparable procedures.

We carried out PLA2 activity assays in triplicate using a continuous fluorescent measurement. In a 96-well microplate, we diluted 5 μL lumbar CSF (or 10 μmol/L recombinant sPLA2 or 5 μL of appropriately diluted serum) in 90 μL PLA2 assay buffer (10 mmol/L Tris-HCl, pH 7.4, 100 mmol/L KCl, 5 mmol/L $CaCl_2$, 1 mmol/L dithiothreitol). We added 5 μL bis-Bodipy FL C_{11}-PC-labeled 100% DOPC liposomes to each well and immediately placed the microplate in a temperature controlled (30 °C) cytofluor multiwell plate reader series 4000 (PerSeptive Biosystems). The fluorescence intensity was recorded over 90 min (91 cycles of 60 s each) at 485 nm excitation and 530 nm emission. Finally, we evaluated PLA2 activity using linear curve fitting with Graph Prism 3.0 (GraphPad).

METHOD VALIDATION

We assessed the linearity of the assay by measuring in triplicate the PLA2 activity at 5 different concentrations of recombinant sPLA2 (0–2 μmol/L) as well as 5 different CSF volumes (0–10 μL). We estimated the precision by measuring repeatability and intermediate precision. We assessed both measurements with 3 different concentrations of recombinant sPLA2 (0.25, 0.5, and 1 μmol/L) as well as with 3 different CSF volumes (2.5, 5, and 7.5 μL). We calculatedc repeatability by measuring PLA2 activity in sextuplicate. To evaluate intermediate precision, we analyzed each sample 3 times a day on 3 consecutive days.

PLA2 INHIBITION

We prepared stock solutions and serial dilutions of PLA2 inhibitors (AACOCF3, BEL, and thioetheramide-PC) in PLA2 assay buffer supplemented by 4.25 mmol/L Triton X-100. Each assay was carried out in the absence or presence of 5 μL of the appropriate inhibitor.

STATISTICAL ANALYSES

We performed statistical analyses with Statgraphics Centurion XV (StatPoint) and Graph Prism 3.0 (GraphPad). We considered variable distributions nonnormal when values of skewness and kurtosis were outside the range −2 to +2. Non–normally distributed variables were analyzed by nonparametric tests or were log-transformed before parametric tests. We compared frequency distributions using the Fisher test. We assessed differences between 2 means using unpaired, 2-tailed Student *t*-test or Mann–Whitney test. Pearson correlation coefficients were calculated for statistical analysis of correlation. The level of significance was defined as $P < 0.05$. Results are expressed as mean (SD).

Results

DEVELOPMENT AND VALIDATION OF THE PLA2 ACTIVITY ASSAY

To measure PLA2 activity in human CSF, we developed a continuous fluorescence assay using an already well-known PLA2 specific substrate, self-quenched fluorescent bis-Bodipy FL C_{11}-PC (9). This substrate (because of its 2 Bodipy fluorophores added to the *sn*-1 and *sn*-2 acyl chains of phosphatidylcholine), once incorporated into artificial cell membranes such as liposomes, exhibits attenuated fluorescence due to energy transfer between the 2 Bodipy fluorophores. On cleavage by PLA2, the Bodipy from the *sn*-2 acyl chain is released and an increase in fluorescence results from the separation of the 2 Bodipy moieties. Because PLA2 activity has been reported to depend on phospholipid concentration and composition from liposomes (9), we assessed the effect of these 2 parameters on CSF as well as on recombinant sPLA2 activities to optimize the conditions for measurement. We observed a maximum activity of the enzymatic reaction, for CSF as well as recombinant sPLA2, when using liposomes made from 20 μg/mL 100% DOPC and labeled with 5 μmol/L bis-Bodipy FL C_{11}-PC (Fig. 1). These conditions allowed us to record a time-dependent increase in fluorescence intensity (FI) from lumbar CSF (Fig. 2A), whereas bis-Bodipy FL C_{11}-PC–labeled 100% DOPC liposomes alone did not show such increases. Because no artifactual change in FI was associated with the substrate, the observed changes in FI during the reaction can be associated with artifactual hydrolysis of bis-Bodipy FL C_{11}-PC by CSF PLA2, which produces fluorescence-labeled free fatty acid and lysophosphatidylcholine. Similar results were obtained with recombinant sPLA2 (data not shown).

In line with the recommendations of the International Conference on Harmonisation guideline on validation of analytical procedures (10), we validated this new continuous fluorescence assay using both recombinant sPLA2 (Fig. 2B) and lumbar CSF (Fig. 2C and D). The assay imprecision was <15% of CV, which meets the recommended guidelines (11). The CV range for imprecision was 2.0%–14.8% for recombinant sPLA2 in concentrations 0.25–1 μmol/L and 1.8%–13.4% for 2 different CSF samples with sizes ranging from 2.5 to 7.5 μL per assay. Moreover, the initial rate of reaction depended on recombinant sPLA2 concentration (Fig. 2B) and CSF volume (Fig. 2C and D), giving a recombinant sPLA2 signal-to-concentration linearity over the range 0.25–1.5 μmol/L ($r^2 = 0.999$, $P < 0.001$) and a linear signal-to-CSF volume relationship over the range 2.5–10 μL for the CSF1 ($r^2 = 0.997$, $P = 0.001$) as well as for the CSF2 ($r^2 = 0.992$, $P = 0.004$) tested.

CHARACTERIZATION OF THE CSF PLA2 ACTIVITY

Although 22 different PLA2 enzymes classified in 5 families (sPLA2, cPLA2, iPLA2, PAF-AH, and lysosomal PLA2) have been identified (1), no specific tool allowing the specific characterization of each of these isoenzyme is available to date. Thus, with regard to a new PLA2 activity identification, the current approach is to determine the family instead of the concerned specific isoenzyme. For this purpose, a battery of inhibitors more or less specific to each PLA2 family is generally used to discriminate 1 family from another. Thus, to identify the type of PLA2 activity in the CSF measured using our assay, we performed incubation of 5 μL lumbar CSF in the presence of 3 different PLA2 inhibitors (AACOCF3, BEL, and thioetheramide-PC) that are known to exhibit different relative potencies for the 3 major families of the enzyme (sPLA2, cPLA2, and iPLA2). Indeed, whereas AACOCF3 is a selective inhibitor of both cPLA2 and iPLA2 (12,13), BEL inhibits specifically isoenzymes from the iPLA2 family (12), and thioetheramide-PC functions as a competitive inhibitor toward sPLA2 (14). The pharmacological profile of the PLA2 activity in lumbar CSF was found to be most consistent with the sPLA2 subtype (Fig. 3). Indeed, wheres the sPLA2-specific inhibitor thioetheramide-PC dose-dependently inhibited the PLA2 activity from lumbar CSF as well as from the recombinant sPLA2 (Fig. 3C), the 2 other inhibitors failed to do so (Fig. 3A and B).

Although calcium is known to be an absolute requirement for the activity of both sPLA2 and cPLA2, its role in the 2 activations is different. Calcium resides at the catalytic center of sPLA2 and is directly involved in substrate–enzyme interaction (15), whereas it is not required for cPLA2 catalytic activity but rather involved in the translocation of cPLA2 from cytosol to the membrane where the substrates are located (16). Thus, to further characterize the CSF PLA2 and to confirm its type, we assessed PLA2 activity in the presence of various concentrations of calcium. With increase in the concentration of calcium, we observed an increase of PLA2 activity in lumbar CSF as well as recombinant sPLA2 (Fig. 3D). Thus, the PLA2 activity measured in human CSF appears to be mainly sPLA2 activity. The PLA2 activity observed in CSF was unlikely to be due to either PAF-AH or lysosomal PLA2, since both of them are calcium-independent (17,18).

MEASUREMENT OF sPLA2 ACTIVITY IN NORMAL CSF

To determine whether CSF sPLA2 activity depends on age or sex, we assessed its normal physiologic levels from 32 healthy volunteers (16 women and 16 men; age 52.3 (15.2) years; MMSE score 29.5 (0.5); sPLA2 activity 3.8 (1.0) ΔFI/min). As shown in Fig. 4A, sPLA2 activity in CSF does not seem to correlate with age. Moreover, no significant difference in sPLA2 activity was noted between men and women (Fig. 4B).

CSF sPLA2 ACTIVITY VS SERUM sPLA2 ACTIVITY

To better understand the origin of the sPLA2 activity measured in lumbar CSF, we simultaneously assessed sPLA2 activity in 10 undiluted lumbar CSFs as well as diluted sera from the same cases (Table 1). We found that the sPLA2 activity relative to protein concentration was 2.5-fold higher in serum than in lumbar CSF which, based on the basic feature of both brain- and blood-derived proteins (19), seems to be of brain origin with a blood-derived fraction in it.

CSF sPLA2 ACTIVITY IN ALZHEIMER DISEASE

Evidence from epidemiological, clinical, and experimental studies supports the hypothesis that inflammatory events are part of the neuropathology of AD (20,21). Several markers of neuroinflammation have been measured in biological fluids, but their specificity for AD remains controversial (22). To assess the relevance of CSF sPLA2 activity measurement as a

neuroinflammatory biomarker for AD, we performed its measurement in CSF from 33 AD patients and 19 age-matched controls. As shown in Table 2, a significant increase in CSF sPLA2 activity was noted for AD compared with the control group.

Discussion

We have developed a simple continuous fluorescence assay for measuring PLA2 activity in CSF using a 96-well microplate format that allows for high-throughput analysis of samples. In addition to fluoro-metric assays, titrimetric, colorimetric, and radiometric methods can be used to determine PLA2 activity in biological fluids (23). With the exception of the radiometric, however, these methods are not sufficiently sensitive. Although radiometric methods are more sensitive, they are hazardous, laborious, and impractical for assaying large numbers of samples. Thus, albeit with a sensitivity that is second to radiometric methods (23), fluorescence-based assays offer the advantage of being suitable for the analysis of a large number of biological fluid samples in a clinical setting.

The performance of a fluorescence-based assay is highly dependent on the conditions and in particular on the type of fluorescent PLA2 substrate used. Several substrates, differentiated by type, number, and localization of the fluorophores, are commercially available. The 2 more frequently used substrates are NBD C6-HPC® [2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine] and bis-Bodipy FL C_{11}-PC. The former exhibits 1 fluorophore on the *sn*-2 phospholipid acyl chain, whereas the latter exhibits an additional fluorophore on the *sn*-1 phospholipid acyl chain, which contributes to its self-quenching. When using NBD C6-HPC, an additional laborious step of separation of the remainder substrate from the product is required (24). In the case of bis-Bodipy FL C_{11}-PC, this step is not required (9), allowing the continuous monitoring of PLA2 activity. When combined, these characteristics make our approach the most attractive methodology currently available.

We also show that the use of liposomes made up of 20 μg/mL phospholipids containing 100% DOPC and labeled with 5 μmol/L bis-Bodipy FL C_{11}-PC as substrate in 10 mmol/L Tris-HCl (pH 7.4) supplemented by 5 mmol/L $CaCl_2$ is optimal to measure PLA2 activity in 5 μL lumbar CSF per assay. The linear signal-to-CSF volume relationship over the range 2.5–10 μL CSF demonstrates the sensitivity of this approach. Another strength of our assay is its reliability as shown by CV <15% for repeatability and intermediate precisions (11).

In the present study, for the first time, we identified the secretory Ca^{2+}-dependent PLA2 activity in lumbar CSF. The observations that 3 mmol/L thioetheramide-PC inhibited >60% of substrate cleavage and that the FI increase was calcium dependent suggest that the CSF activity is predominantly due to sPLA2 type. Additionally, negative results from the pharmacologic experiments using AACOCF3 and BEL, 2 inhibitors of cPLA2/iPLA2 and iPLA2, respectively, confirm this conclusion. Based on our data, however, we cannot identify which specific isoenzymes give rise to this sPLA2 activity. The identification of sPLA2 isoenzymes from CSF using selective antibodies should be considered. At present, however, the lack of availability of specific antibodies against each sPLA2 isoenzyme makes this identification difficult. In addition, we cannot rule out the presence of other PLA2 types in human CSF. To our knowledge, only 1 other study has reported on this subject to date (25); but the authors of that study claimed that the PLA2 activity identified in their study was most likely from cPLA2 and iPLA2 types. Thus, because the PLA2 type targeted in a specific assay in part depends on the methodology used, we can expect the future identification of other CSF PLA2 types as has been seen in studies of human serum using independent methodologies (9,24,26).

Phospholipase A_2 enzymes are well known to play a role in neuroinflammation (2). Concerning the specific secretory Ca^{2+}-dependent PLA2 family, a large variety of additional biological functions have been proposed. These include, among others, reactive oxygen species generation (27), apoptosis (28), and cell proliferation (29). To date, however, the in vivo biological functions, in physiological as well as pathological conditions, of each brain sPLA2 remain unknown. We believe that the development of this simple and sensitive assay as well as the demonstration of the secretory (extracellular) Ca^{2+}-dependent PLA2 activity in CSF will stimulate studies that will enhance the understanding of the role of these isoenzymes in neurological disorders.

The discovery of the sPLA2 activity in lumbar CSF allows us to envisage the use of our assay in the diagnosis of neurological disorders associated with inflammation. Such a consideration raises the question of whether an increase of sPLA2 activity in CSF reflects the cause or the outcome of a neuroinflammatory process. In other words, is the sPLA2 activity measured in lumbar CSF of central nervous system origin or of blood origin or both? Because sPLA2 activity is present in both serum (9) and brain (2), both origins can be considered equally. The simultaneous measurement of sPLA2 activity in both CSF and serum from the same cases, at equal protein concentrations, revealed a CSF: serum sPLA2 activity ratio of 1:2.5. In considering the basic features of both brain- and blood-derived proteins as described by Reiber (19), such a ratio is consistent with a brain origin associated with a blood-derived fraction of the enzyme activity. Furthermore, the fact that the CSF sPLA2 activity did not increase from age 20 to 77 whereas the CSF/serum albumin ratio does increase with age (30), also supports the main brain origin of the CSF sPLA2 activity we have observed. However, the discrimination between brain and blood origin is difficult to assess once the multiplicity of serum and brain PLA2 isoenzymes characterized by a sPLA2 activity is taken into consideration.

The presence of activated glial cells and the increase in inflammation-associated proteins in AD brain had led to the consideration that chronic inflammation plays a role in the pathophysiology of AD (20,21). For these reasons, several mediators of inflammation such as interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α) have been extensively studied in CSF of AD patients, but conflicting results have been observed (22). Indeed, although the majority of studies report no difference (31,32), others showed an increase (33,34) or even a decrease (35) in levels of these inflammatory markers in CSF of AD patients compared with controls. Due to this low specificity, the use of cytokines as biomarkers of AD is questionable. Other key molecules involved in neuroinflammation, such as constituents of the arachidonic acid cascade, have been investigated in the CSF. The downstream events of this pathway result in enzymatic oxidation by cyclooxygenase and lipoxygenase and in the nonenzymatic peroxidation of arachidonic acid (AA) in proinflammatory eicosanoids (i.e., prostaglandins, leukotrienes, and thromboxanes) and isoprostanes, respectively (36,37). The concentrations of eicosanoids and isoprostanes in CSF are of particular interest in the biomarker field, and increases in CSF prostaglandin E_2 (PGE2) and F_2-isoprostane have been reported in AD patients (38,39). However, such analyses require highly specialized equipment and expertise, e.g., GC/MS, and, the measurements of CSF PGE2 and F_2-isoprostane have not, to date, been widely used in practice. The upstream events of the arachidonic acid cascade corresponding to AA synthesis by PLA2 are of major interest in neuroinflammation. Whereas the quantification of ^{11}C-AA incorporation into human brain by positron emission tomography has provided promising results (40), little is known about phospholipase A_2 activity in CSF. In this study, we have shown for the first time a significant increase of sPLA2 activity in AD compared with age-matched controls, which may allow consideration of such measurement as a neuroinflammatory biomarker.

In conclusion, we have developed a new continuous fluorescence method using commercially available reagents to measure the CSF activity of a well-known inflammatory key enzyme, i.e., the secretory (extracellular) Ca^{2+}-dependent PLA2. We have also demonstrated that this microplate assay meets several of the ideal biomarker requirements such as reliability, reproducibility, and simplicity to perform (41). The discovery of the sPLA2 activity in human lumbar CSF should facilitate investigations of this enzyme activity in CSF from a variety of neurological diseases, especially neurodegenerative and neuroinflammatory disorders. The increase in the CSF level of sPLA2 activity in Alzheimer disease compared with age-matched healthy controls discovered in the present study is consistent with the involvement of neuroinflammation in this disease.

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References

1. Burke JE, Dennis EA. Phospholipase A(2) Biochemistry. Cardiovasc Drugs Ther 2009;23:49–59. [PubMed: 18931897]
2. Farooqui AA, Horrocks LA. Phospholipase A_2-generated lipid mediators in the brain: the good, the bad, and the ugly. Neuroscientist 2006;12:245–60. [PubMed: 16684969]
3. Farooqui AA, Ong WY, Horrocks LA. Inhibitors of brain phospholipase A_2 activity: their neuropharmacological effects and therapeutic importance for the treatment of neurologic disorders. Pharmacol Rev 2006;58:591–620. [PubMed: 16968951]
4. Nicholson B, Leach CA, Goldenberg SJ, Francis DM, Kodrasov MP, Tian X, et al. Characterization of ubiquitin and ubiquitin-like-protein isopeptidase activities. Protein Sci 2008;17:1035–43. [PubMed: 18424514]
5. Morioka Y, Saiga A, Yokota Y, Suzuki N, Ikeda M, Ono T, et al. Mouse group X secretory phospholipase A_2 induces a potent release of arachidonic acid from spleen cells and acts as a ligand for the phospholipase A_2 receptor. Arch Biochem Biophys 2000;381:31–42. [PubMed: 11019817]
6. McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. Neurology 1984;34:939–44. [PubMed: 6610841]
7. Folstein MF, Folstein SE, McHugh PR. "Mini-mental state": a practical method for grading the cognitive state of patients for the clinician. J Psychiatr Res 1975;12:189–98. [PubMed: 1202204]
8. Kataoka S, Paidi M, Howard BV. Simplified isoelectric focusing/immunoblotting determination of apoprotein E phenotype. Clin Chem 1994;40:11–3. [PubMed: 8287516]
9. Tsao FH, Shanmuganayagam D, Zachman DK, Khosravi M, Folts JD, Meyer KC. A continuous fluorescence assay for the determination of calcium-dependent secretory phospholipase A_2 activity in serum. Clin Chim Acta 2007;379:119–26. [PubMed: 17292873]
10. ICH Harmonised Tripartite Guideline—Validation of Analytical Procedures: Text and Methodology Q2(R1); International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 2005.
11. Food and Drug Administration. Guidance for Industry: Bioanalytical Method Validation. 2001. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf (Accessed October 14, 2009)

12. Ackermann EJ, Conde-Frieboes K, Dennis EA. Inhibition of macrophage Ca(2+)-independent phospholipase A_2 by bromoenol lactone and trifluoromethyl ketones. J Biol Chem 1995;270:445–50. [PubMed: 7814408]
13. Street IP, Lin HK, Laliberte F, Ghomashchi F, Wang Z, Perrier H, et al. Slow- and tight-binding inhibitors of the 85-kDa human phospholipase A_2. Biochemistry 1993;32:5935–40. [PubMed: 8018213]
14. Plesniak LA, Boegeman SC, Segelke BW, Dennis EA. Interaction of phospholipase A_2 with thioether amide containing phospholipid analogues. Biochemistry 1993;32:5009–16. [PubMed: 8494876]
15. Scott DL, White SP, Browning JL, Rosa JJ, Gelb MH, Sigler PB. Structures of free and inhibited human secretory phospholipase A_2 from inflammatory exudate. Science (Wash DC) 1991;254:1007–10.
16. Nalefski EA, Sultzman LA, Martin DM, Kriz RW, Towler PS, Knopf JL, Clark JD. Delineation of two functionally distinct domains of cytosolic phospholipase A_2, a regulatory Ca(2+)-dependent lipid-binding domain and a Ca(2+)-independent catalytic domain. J Biol Chem 1994;269:18239–49. [PubMed: 8027085]
17. Abe A, Shayman JA. Purification and characterization of 1-*O*-acylceramide synthase, a novel phospholipase A_2 with transacylase activity. J Biol Chem 1998;273:8467–74. [PubMed: 9525960]
18. Stafforini DM. Biology of platelet-activating factor acetylhydrolase (PAF-AH, lipoprotein associated phospholipase A(2)). Cardiovasc Drugs Ther 2009;23:73–83. [PubMed: 18949548]
19. Reiber H. Dynamics of brain-derived proteins in cerebrospinal fluid. Clin Chim Acta 2001;310:173–86. [PubMed: 11498083]
20. McGeer PL, McGeer EG. The inflammatory response system of brain: implications for therapy of Alzheimer and other neurodegenerative diseases. Brain Res Brain Res Rev 1995;21:195–218. [PubMed: 8866675]
21. McGeer PL, Rogers J. Anti-inflammatory agents as a therapeutic approach to Alzheimer's disease. Neurology 1992;42:447–9. [PubMed: 1736183]
22. Teunissen CE, de Vente J, Steinbusch HW, De Bruijn C. Biochemical markers related to Alzheimer's dementia in serum and cerebrospinal fluid. Neurobiol Aging 2002;23:485–508. [PubMed: 12009495]
23. Reynolds LJ, Washburn WN, Deems RA, Dennis EA. Assay strategies and methods for phospholipases. Methods Enzymol 1991;197:3–23. [PubMed: 2051923]
24. Smesny S, Kinder D, Willhardt I, Rosburg T, Lasch J, Berger G, Sauer H. Increased calcium-independent phospholipase A_2 activity in first but not in multiepisode chronic schizophrenia. Biol Psychiatry 2005;57:399–405. [PubMed: 15705356]
25. Smesny S, Stein S, Willhardt I, Lasch J, Sauer H. Decreased phospholipase A_2 activity in cerebrospinal fluid of patients with dementia. J Neural Transm 2008;115:1173–9. [PubMed: 18584113]
26. Kosaka T, Yamaguchi M, Miyanaga K, Mizuno K. Serum platelet-activating factor acetylhydrolase (PAF-AH) activity in more than 3000 healthy Japanese. Clin Chim Acta 2001;312:179–83. [PubMed: 11580924]
27. Muralikrishna AR, Hatcher JF. Phospholipase A_2, reactive oxygen species, and lipid peroxidation in cerebral ischemia. Free Radic Biol Med 2006;40:376–87. [PubMed: 16443152]
28. Taketo MM, Sonoshita M. Phospolipase A_2 and apoptosis. Biochim Biophys Acta 2002;1585:72–6. [PubMed: 12531539]
29. Masuda S, Yamamoto K, Hirabayashi T, Ishikawa Y, Ishii T, Kudo I, Murakami M. Human group III secreted phospholipase A_2 promotes neuronal outgrowth and survival. Biochem J 2008;409:429–38. [PubMed: 17868035]
30. Reiber H. Proteins in cerebrospinal fluid and blood: barriers, CSF flow rate and source-related dynamics. Restor Neurol Neurosci 2003;21:79–96. [PubMed: 14530572]
31. Galimberti D, Venturelli E, Fenoglio C, Guidi I, Villa C, Bergamaschini L, et al. Intrathecal levels of IL-6, IL-11 and LIF in Alzheimer's disease and frontotemporal lobar degeneration. J Neurol 2008;255:539–44. [PubMed: 18204920]
32. Lanzrein AS, Johnston CM, Perry VH, Jobst KA, King EM, Smith AD. Longitudinal study of inflammatory factors in serum, cerebrospinal fluid, and brain tissue in Alzheimer disease:

interleukin-1beta, interleukin-6, interleukin-1 receptor antagonist, tumor necrosis factor-alpha, the soluble tumor necrosis factor receptors I and II, and alpha1-antichymotrypsin. Alzheimer Dis Assoc Disord 1998;12:215–27. [PubMed: 9772027]

33. Blum-Degen D, Muller T, Kuhn W, Gerlach M, Przuntek H, Riederer P. Interleukin-1 beta and interleukin-6 are elevated in the cerebrospinal fluid of Alzheimer's and de novo Parkinson's disease patients. Neurosci Lett 1995;202:17–20. [PubMed: 8787820]
34. Tarkowski E, Blennow K, Wallin A, Tarkowski A. Intracerebral production of tumor necrosis factor-alpha, a local neuroprotective agent, in Alzheimer disease and vascular dementia. J Clin Immunol 1999;19:223–30. [PubMed: 10471976]
35. Yamada K, Kono K, Umegaki H, Yamada K, Iguchi A, Fukatsu T, et al. Decreased interleukin-6 level in the cerebrospinal fluid of patients with Alzheimer-type dementia. Neurosci Lett 1995;186:219–21. [PubMed: 7777201]
36. Farooqui AA, Horrocks LA, Farooqui T. Modulation of inflammation in brain: a matter of fat. J Neurochem 2007;101:577–99. [PubMed: 17257165]
37. Nourooz-Zadeh J. Key issues in F2-isoprostane analysis. Biochem Soc Trans 2008;36:1060–5. [PubMed: 18793189]
38. Montine TJ, Sidell KR, Crews BC, Markesbery WR, Marnett LJ, Roberts LJ, Morrow JD. Elevated CSF prostaglandin E2 levels in patients with probable AD. Neurology 1999;53:1495–8. [PubMed: 10534257]
39. Montine TJ, Beal MF, Robertson D, Cudkowicz ME, Biaggioni I, O'Donnell H, et al. Cerebrospinal fluid F2-isoprostanes are elevated in Huntington's disease. Neurology 1999;52:1104–5. [PubMed: 10102447]
40. Esposito G, Giovacchini G, Liow JS, Bhattacharjee AK, Greenstein D, Schapiro M, et al. Imaging neuroinflammation in Alzheimer's disease with radiolabeled arachidonic acid and PET. J Nucl Med 2008;49:1414–21. [PubMed: 18703605]
41. The Ronald and Nancy Reagan Research Institute of the Alzheimer's Association and National Institute on Aging Working Group. Consensus Report of the Working Group on Molecular and Biochemical Markers of Alzheimer's Disease. Neurobiology of Aging 1998;19:109–16. [PubMed: 9558143]

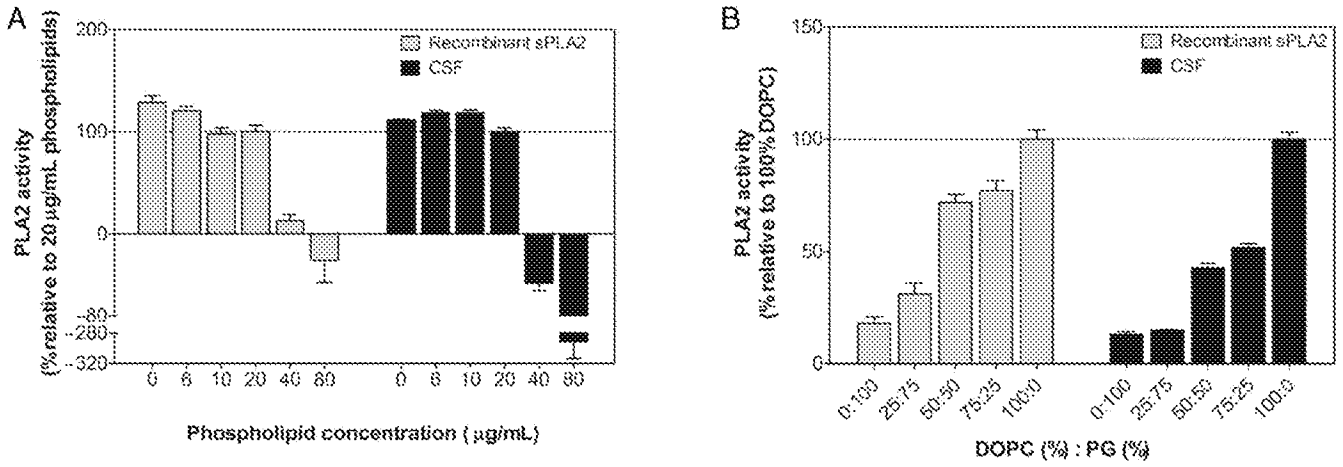

Fig. 1. Effect of the liposome phospholipid concentration and composition on recombinant and lumbar CSF PLA2 activities

(A), Effect of increasing the liposome phospholipid concentration on the rate of the initial reaction of 0.5 μmol/L recombinant sPLA2 or 5 μL CSF. The activity was determined using Bodipy-labeled liposomes composed of 50% DOPC and 50% PG in 10 mmol/L Tris-HCl (pH 7.4) supplemented by 5 mmol/L $CaCl_2$. (B), Effect of varying proportions of DOPC and PG on the rate of the initial reaction of 0.5 μmol/L recombinant sPLA2 and 5 μL CSF. The activity was determined using Bodipy-labeled liposomes made up of 20 μg/mL phospholipids in 10 mmol/L Tris-HCl (pH 7.4) supplemented by 5 mmol/L $CaCl_2$. Data are expressed as mean (SD) (n = 3).

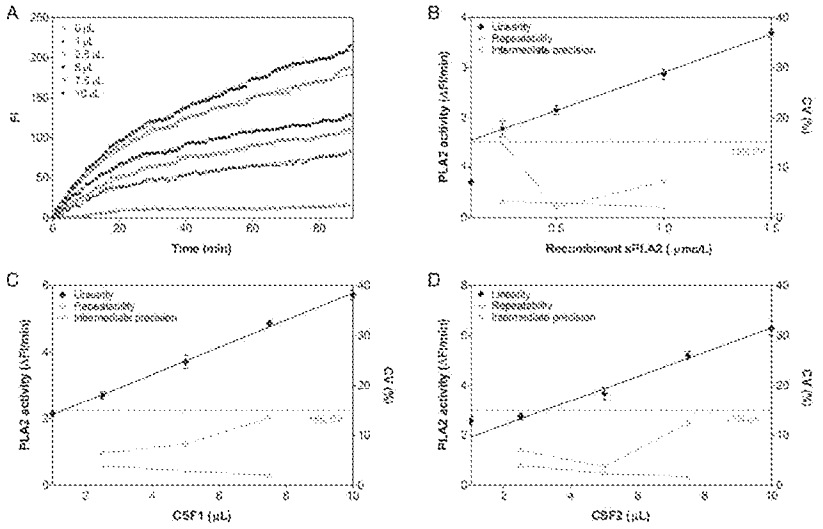

Fig. 2. Linearity and precision profiles for recombinant and lumbar CSF PLA2 activities

(A), Time dependence of the PLA2 activity in a representative experiment using different volumes of lumbar CSF (0–10 μL). Data are expressed as mean of 3 independent experiments. (B–D), Signal-to-concentration linearity performance and CV for repeatability and intermediate precision assessment for recombinant sPLA2 (B) and CSFs from 2 different cases (C and D). Linearity data are shown as mean (SD) (n = 3), and precision are shown as CV resulting from 6 consecutive replicates for repeatability and from 3 measurements on 3 consecutive days (n = 9) for intermediate precision.

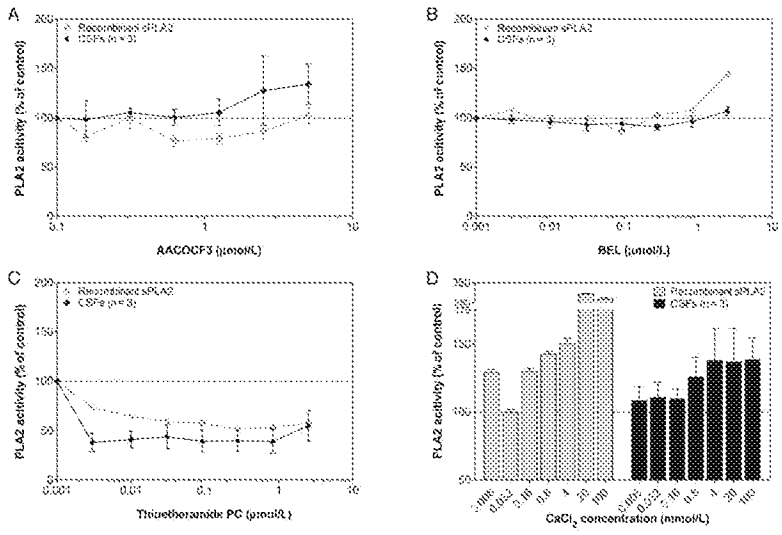

Fig. 3. Effect of PLA2 family-specific inhibitors on recombinant and lumbar CSF PLA2 activities

(A–C), Effect of different concentrations of 3 PLA2 inhibitors: 0.16–5 μmol/L AACOCF3 (A); 0.003–2.5 μmol/L BEL (B); 0.003–2.5 μmol/L thioetheramide-PC (C). (D), Effect of increasing concentration of calcium (6.4 μmol/L to 100 mmol/L). PLA2 activity is expressed as percentage of the activity relative to the same sample without any inhibitor or any calcium supplementation. Recombinant sPLA2 and lumbar CSF data are expressed as mean (SD) from 3 experiments and from 3 different CSFs each analyzed in triplicate, respectively.

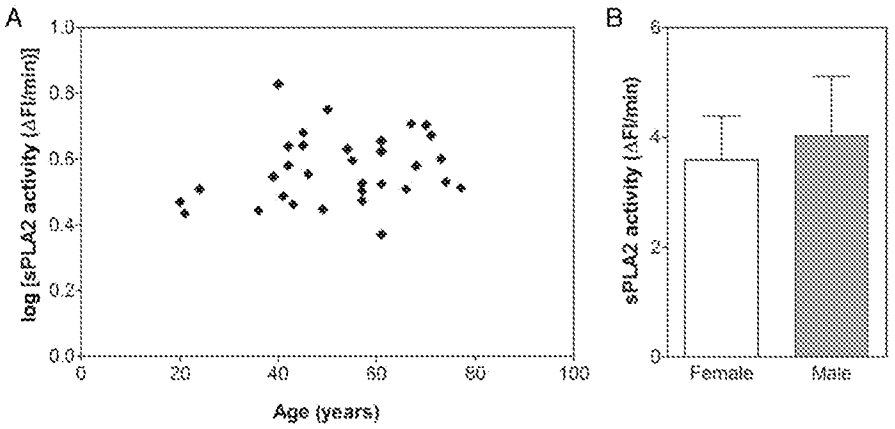

Fig. 4. CSF sPLA2 activity as function of age and sex

(A), Pearson correlation between log(sPLA2 activity) and age ($r = 0.214$; $P = 0.239$; 95% CI −0.145 to 0.524). (B), No significant difference between healthy women and healthy men for sPLA2 activity ($t_{df=30} = 1.312$; $P = 0.200$). Data are shown as mean (SD).

Table 1

CSF vs serum sPLA2 activity.[a]

	Healthy individuals (n = 10)
Sex, F/M	3/7
Age, years	53.0 (22.6)
CSF/serum sPLA2 activity ratio[b]	0.4 (0.1)
CSF/serum protein ratio[c]	0.013 (0.003)

[a] Categorical data (sex) and continuous variables (age, CSF/serum sPLA2 activity ratio, CSF/serum protein ratio) are expressed as number of individuals and as mean (SD), respectively.

[b] Whereas CSFs were undiluted, sera were appropriately diluted to get similar protein concentration between CSF and serum-matched samples.

[c] Protein concentrations were measured using bicinchoninic acid protein assay (Pierce).

Table 2

Demographic and clinical features and CSF levels of sPLA2 activity of AD and age-matched controls.[a]

	Control	AD	Statistic value	*P*
	19	33		
Male sex, n (%)	12 (63)	17 (52)	NA	0.564
Age, years	62.6 (8.2)	66.8 (3.6)	$z = 212.5$	0.055
MMSE	29.5 (0.5)	22.7 (6.4)	$z = 58.5$	0.0008
ApoE4 carrier, n (%)	0 (0)	19 (58)	NA	<0.0001
sPLA2 activity, ΔFI/min	3.8 (0.9)	5.1 (1.3)	$t_{df=50} = 3.59$	0.0008

[a]Differences between groups were assessed using Fisher test. Continuous variables are expressed as mean (SD), and differences between groups were assessed using Mann–Whitney test (age, MMSE) or 2-tailed Student *t*-test (sPLA2 activity). NA, not applicable.

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Short Communication

Genetic Deficiency of Plasma Lipoprotein-Associated Phospholipase A_2 (*PLA2G7* V297F Null Mutation) and Risk of Alzheimer's Disease in Japan

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Abstract. High plasma lipoprotein phospholipase A_2 activity (Lp-PLA_2) is reported to be a risk factor for dementia. A loss of function polymorphism in the Lp-PLA_2 gene – *PLA2G7* V279F – is found almost exclusively in Asians. In 1,952 subjects with late-onset AD and 2,079 non-demented controls recruited from Japan, the *PLA2G7* null allele was not associated with risk or age at onset of AD: logistic regression OR 0.98 (95% CI 0.86–1.12, $p = 0.81$) per additional null allele, adjusted for age/age at onset, gender, and *APOE* $\varepsilon 4$. Genetic deficiency of Lp-PLA_2 activity is not associated with a reduced risk of AD in the Japanese population.

Keywords: Alzheimer's disease, apolipoprotein E, genetic association study, lipoprotein-associated phospholipase A2, Mendelian randomization

INTRODUCTION

Cardiovascular risk factors such as hypertension, hyperlipidemia, and diabetes are implicated as risk factors for Alzheimer's disease (AD) [1]. Lipoprotein-associated phospholipase A_2 (Lp-PLA_2) is an enzyme produced by inflammatory cells which is believed to contribute to atherogenesis in humans [2]. Numerous epidemiologic studies suggest that high levels of circulating Lp-PLA_2 are associated with cardiovascular events and stroke [3–5]. In the Rotterdam case-cohort study, high Lp-PLA_2 activity was also independently associated with the risk of developing dementia (HR = 1.56 for highest quartile relative to lowest; 95% CI 1.03–2.37); the effect was greater for vascular dementia

[1]Equal contribution.

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(HR = 2.19; CI 0.80–6.03) than for AD (HR = 1.30; CI 0.82–2.04) [6].

A genetic polymorphism in the Lp-PLA$_2$ gene (*PLA2G7*), 994G>T (V279F), termed the Asian null mutation, is found almost exclusively in Asians and is associated with absence of plasma enzyme activity [7–9]. Heterozygous carriers of the null mutation have 50% of the normal enzyme activity in plasma; null homozygotes have no enzyme activity in plasma [10]. In Japan, the frequency of heterozygous carriers (GT genotype) is ~25%, and of null homozygotes (TT genotype) is ~2–3%, corresponding to a minor allele frequency of ~15% [11]. The presence in Japanese individuals of this relatively common polymorphism strongly correlated to Lp-PLA$_2$ activity allowed us to perform a Mendelian randomization experiment, using the *PLA2G7* null polymorphism as an instrument to test whether lifelong genetic deficiency of Lp-PLA$_2$ activity protects against AD.

METHODS

Study population

The subjects in this study are from the Japanese Genetic Study Consortium for AD (JGSCAD) [12–15]. AD patients (n = 1,952) were recruited from neurology clinics throughout Japan and satisfied NINCDS-ADRDA criteria for probable AD after clinical, cognitive, and imaging evaluation (MRI, FDG-PET) [16]. Vascular dementia, frontotemporal dementia, and other neurological degenerative disorders were excluded. Age at onset was based on the first symptoms of cognitive impairment reported by the patient's spouse or caregiver. Control subjects (n = 2,079) were recruited from the local community, had no signs of dementia, and lived in an unassisted manner. The Mini-Mental State Examination (MMSE), Clinical Dementia Rating (CDR), and Functional Assessment Staging (FAS) were used to assess presence and severity of cognitive impairment in cases and controls [17–19].

The JGSCAD was approved by the Institutional Review Board (IRB) of the University of Niigata and by all participating institutes. Informed consent was obtained from all controls and appropriate proxies for patients. The *PLA2G7* study was approved by the University of Niigata IRB. All samples were anonymously analyzed for genotyping.

Genotyping

Genomic DNA preparation and genotyping were described previously [12]. The *PLA2G7* V279F (RS16874954) variant and three other missense polymorphisms – R92H (RS1805017), I198T (RS1805018), and A379V (RS1051931) – which may have an impact on Lp-PLA$_2$ activity were genotyped by Taqman [20].

Statistical analysis

The SNP and Variation SuiteTM V7.2.1 (Golden Helix) Fisher's Exact Test was used to assess Hardy Weinberg Equilibrium (HWE). Logistic regression using an additive genetic model estimated the effect of each *PLA2G7* variant on case-control status, adjusted for age (age of controls, age at onset for cases), gender, and number of *APOE* ε4 alleles (0, 1, 2). Cox Proportional Hazards regression assessed the effect of each variant on age at onset, adjusted for gender and *APOE* (SAS v9.1.3); controls were censored at the age individuals entered the study. Additional analyses were conducted stratified by gender and by *APOE* ε4 carriage (*APOE* ε4 positive or *APOE* ε4 negative).

The study had 80% power to identify an OR of 0.81 per additional V279F allele (additive model) assuming a minor allele frequency of 0.16 and sample size of 1,952 AD subjects and 2,079 controls.

RESULTS

Demographic information for study subjects are presented in Table 1. Mean (SD) age at onset of the AD subjects was 74.1 years (5.0), and 71% were female. Mean age of the control subjects was 75.3 (6.1), and 58% were female. Mean MMSE, CDR, and FAS scores were consistent with mild-moderate dementia in AD subjects, and with absence of dementia in control subjects. As expected, *APOE* ε4 was over-represented in AD subjects: 53% of AD subjects carried the *APOE* ε4 allele, compared to 17% of controls.

The genotype frequencies for each of the *PLA2G7* polymorphisms are presented for AD cases and controls in Table 1. The number of subjects with missing genotypes was small: 26, 31, 38, and 43 for V279F, R92H, I198T, and A379V, respectively. All of the variants were in Hardy-Weinberg equilibrium in both cases and controls. HWE p-values for V279F, R92H, I198T, and A379V in controls were 0.42, 0.65, 0.73, and 0.72 and in cases were 0.61, 0.95, 0.42, and 0.90, respec-

Table 1
Demographics of the study sample including *APOE* ε4 and *PLA2G7* genotype frequencies

Characteristic	AD	Control
N	1,952	2,079
AAO/AAE (years) (mean ± SD)	74.1 ± 5.0	75.3 ± 6.1
Gender (N, %)		
Female	1387 (71.1%)	1198 (57.6%)
Male	565 (28.9%)	881 (42.4%)
MMSE (mean ± SD)	17.2 ± 6.5	28.6 ± 1.5
CDR (mean ± SD)	1.44 ± 0.71	0.004 ± 0.044
FAS (mean ± SD)	4.58 ± 1.06	1.64 ± 0.61
APOE ε4 copies (N, %)		
0	914 (46.8%)	1725 (83.0%)
1	861 (44.1%)	341 (16.4%)
2	177 (9.1%)	13 (0.6%)
PLA2G7 genotypes (N, %)		
V279F (null variant)		
VV	1356 (70.2%)	1448 (69.9%)
VF	530 (27.4%)	575 (27.8%)
FF	47 (2.4%)	49 (2.4%)
R92H		
RR	1183 (61.4%)	1263 (61.0%)
RH	656 (34.0%)	705 (34.0%)
HH	89 (4.6%)	104 (5.0%)
I198T		
II	1249 (64.8%)	1341 (64.9%)
IT	613 (31.8%)	643 (31.1%)
TT	66 (3.4%)	81 (3.9%)
A379V		
AA	1560 (80.9%)	1663 (80.7%)
AV	348 (18.1%)	378 (18.4%)
VV	20 (1.0%)	19 (0.9%)

AD, Alzheimer's disease. AAO, age at onset. AAE, age at examination. MMSE, Mini-Mental State Examination (The MMSE is scored from 0 to 30, with higher scores indicating better cognitive function [17]). CDR, Clinical Dementia Rating (The CDR is scored on an ordinal scale, with 0=no impairment, 0.5 = questionable impairment, 1 = mild impairment, 2 = moderate impairment, 3 = severe impairment [18]). FAST, Functional Assessment Staging (FAST is scored on an ordinal scale from 1 = no objective or subjective functional decrement, to 7 corresponding to severe AD [19]). SE, Standard deviation.

tively. Genotype and allele frequencies did not differ between AD cases and controls by Fisher Exact Test.

The *PLA2G7* 279F null allele had no significant effect on AD risk or age of onset, overall or within gender and *APOE* ε4 sub-groups (Fig. 1). The odds ratio (OR) for the 279F null allele was 0.98 (95% CI 0.86, 1.12) per additional null allele, adjusted for age/age at onset, gender, and number of *APOE* ε4 alleles. The OR was 0.97 (95% CI 0.82, 1.15) in the female subset, and 1.01 (95% CI 0.81, 1.26) in the male subset. Although the OR point estimates in the *APOE* ε4-negative groups trended slightly less than 1, and those in the *APOE* ε4-positive sub-groups trended slightly greater than 1, all 95% CI intervals spanned 1 and nominal p-values were not significant. Hazard ratio (HR) estimates were similar in the age of onset analysis. 297F was not significantly associated with AD risk or age of onset in alternative dominant, co-dominant, and recessive parameterizations of the polymorphism.

The other *PLA2G7* missense polymorphisms (92H, 198T, 379V) had no significant effect on either AD risk or age of onset, with mean OR of 0.94-0.97 per additional allele.

DISCUSSION

Results from the Rotterdam cohort study suggested that plasma Lp-PLA$_2$ activity, implicated in cardiovascular disease and stroke, might also be a risk factor for dementia [6]. However, in this large genetic case-control study, inherited deficiency of Lp-PLA$_2$ activity in Japan did not influence AD susceptibility or age at onset. The *APOE* ε4 allele is a risk factor for both cardiovascular disease and AD, but no *PLA2G7* V297F effect was identified after adjusting for presence or number of *APOE* ε4 alleles, or within strata of *APOE* ε4 carriers and non-carriers. Our data is generated from an ethnically homogenous Japanese population which has the highest population allele frequency of the V279F variant. This variant corresponds to a lifelong 50% deficiency of plasma Lp-PLA$_2$ activity in heterozygotes, and a near absence of enzyme activity in homozygotes.

Genomewide association studies (GWAS) have not implicated PLA2G7 as a risk factor for AD [21–25]; however, most published GWAS were performed in Caucasian populations where the V279F null mutation is not found, and the *PLA2G7* SNP variants examined have minor, if any, functional effects [26]. There was no association of five *PLA2G7* SNPs (RS9472826, RS1421372, RS16874962, RS1421378, RS10948301) with AD diagnosis or age at onset in a GWAS of Canadian AD cases and controls [22]. The particular strength of the current JGSCAD study is the well-powered scientifically-targeted hypothesis, and the evaluation of a polymorphism (V279F) with a large effect on Lp-PLA$_2$ activity, within the population with the highest frequency for that SNP.

Our results complement epidemiological studies of Lp-PLA$_2$ and dementia or stroke. The absence of an effect of genetically-mediated deficiency of Lp-PLA$_2$ activity on AD risk in JGSCAD is generally consistent with the absence of a significant effect of plasma Lp-PLA$_2$ activity on AD-specific risk in the Rotterdam study [6]. In conjunction with epidemiological studies suggesting that elevated Lp-PLA$_2$ is a risk factor for is-

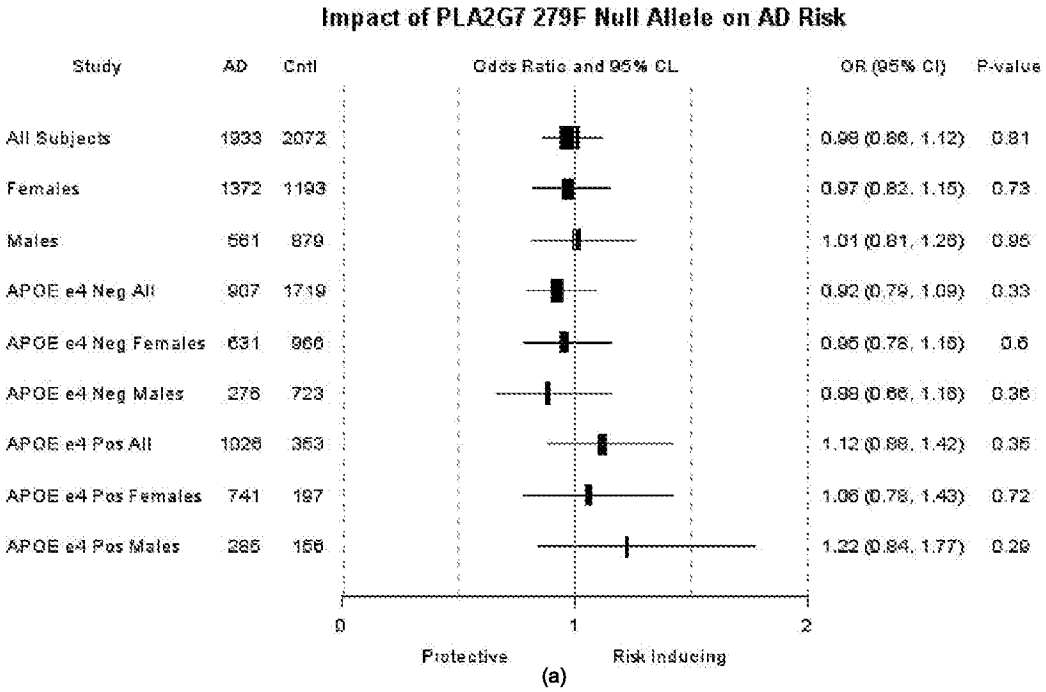

Fig. 1. Odds ratios (95% CI) for association of *PLA2G7* V279F polymorphism with AD status (Fig 1a) and hazard ratios for age at onset (Fig. 1b) overall and within gender and *APOE* ε4 sub-groups. OR = odds ratio; HR = hazard ratio, AD = number of AD cases, Cntl = number of controls.

chemic stroke [5], the genetic and epidemiology results suggest that putative Lp-PLA$_2$ effects on dementia or AD may be mediated by vascular etiologies.

The results of this study should be interpreted in the context of some potential limitations. Firstly, subgroup analyses were based on smaller sample numbers, so power to detect associations in these subsidiary analyses was low. Secondly, the NINCDS-ADRDA criterion for diagnosis of probable AD excludes subjects with clinically significant cerebrovascular disease; therefore, no study conclusions can be made regarding the effect of the *PLA2G7* null polymorphism on vascular dementia. Thirdly, data was not available on covariates of interest such as cardiovascular disease, hypertension, diabetes, or subcortical ischemic changes on imaging that may be associated with *PLA2G7* genotype, limiting evaluation of causal intermediates or effect modification by these co-morbidities. Finally, genetic deficiency of Lp-PLA$_2$ activity could theoretically initiate compensatory mechanisms that alter the risk profile observed in epidemiological studies of Lp-PLA$_2$ activity, and the relationship between Lp-PLA$_2$ activity and AD pathogenesis.

In conclusion, genetic deficiency of Lp-PLA$_2$ activity due to carriage of the V279F null allele is not associated with a reduced risk of AD in Japan.

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REFERENCES

[1] Purnell C, Gao S, Callahan CM, Hendrie HC (2009) Cardiovascular risk factors and incident Alzheimer disease: a systematic review of the literature. *Alzheimer Dis Assoc Disord* **23**, 1-10.

[2] Zalewski A, Macphee C (2005) Role of lipoprotein-associated phospholipase A2 in atherosclerosis: biology, epidemiology, and possible therapeutic target. *Arterioscler Thromb Vasc Biol* **25**, 923-931.

[3] Lp-PLA2 Studies Collaboration, Ballantyne C, Cushman M, Psaty B, Furberg C, Khaw KT, Sandhu M, Oldgren J, Rossi GP, Maiolino G, Cesari M, Lenzini L, James SK, Rimm E, Collins R, Anderson J, Koenig W, Brenner H, Rothenbacher D, Berglund G, Persson M, Berger P, Brilakis E, McConnell JP, Koenig W, Sacco R, Elkind M, Talmud P, Rimm E, Cannon CP, Packard C, Barrett-Connor E, Hofman A, Kardys I, Witteman JC, Criqui M, Corsetti JP, Rainwater DL, Moss AJ, Robins S, Bloomfield H, Collins D, Packard C, Wassertheil-Smoller S, Ridker P, Ballantyne C, Cannon CP, Cushman M, Danesh J, Gu D, Hofman A, Nelson JJ, Thompson S, Zalewski A, Zariffa N, Di Angelantonio E, Kaptoge S, Thompson A, Thompson S, Walker M, Watson S, Wood A (2007) Collaborative meta-analysis of individual participant data from observational studies of Lp-PLA$_2$ and cardiovascular diseases. *Eur J Cardiovasc Prev Rehabil* **14**, 3-11.

[4] Garza CA, Montori VM, McConnell JP, Somers VK, Kullo IJ, Lopez-Jimenez F (2007) Association between lipoprotein-associated phospholipase A2 and cardiovascular disease: a systematic review. *Mayo Clin Proc* **82**, 159-165.

[5] The LP-PLA2 Studies Collaboration (2010) Lipoprotein-associated phospholipase A2 and risk of coronary disease, stroke, and mortality: collaborative analysis of 32 prospective studies. *The Lancet* **375**, 1536-1544.

[6] van Oijen M, van der Meer IM,Hofman A, Witteman JC, Koudstaal PJ, Breteler MM (2006) Lipoprotein-associated phospholipase A2 is associated with risk of dementia. *Ann Neurol* **59**, 139-144.

[7] Hiramoto M, Yoshida H, Imaizumi T, Yoshimizu N, Satoh K (1997) A mutation in plasma platelet-activating factor acetylhydrolase (Val279–>Phe) is a genetic risk factor for stroke. *Stroke* **28**, 2417-2420.

[8] Satoh N, Asano K, Naoki K, Fukunaga K, Iwata M, Kanazawa M, Yamaguchi K (1999) Plasma platelet-activating factor acetylhydrolase deficiency in Japanese patients with asthma. *Am J Respir Crit Care Med* **159**, 974-979.

[9] Yamada Y, Ichihara S, Fujimura T, Yokota M (1998) Identification of the 994C> T missense in exon 9 of the plasma platelet-activating factor acetylhydrolase gene as an independent risk factor for coronary artery disease in Japanese men. *Metabolism* **47**, 177-181.

[10] Zhang SY, Shibata H, Karino K (2007) Comprehensive evaluation of genetic and environmental factors influencing the plasma lipoprotein-associated phospholipase A2 activity in a Japanese population. *Hypertens Res* **30**, 403-409.

[11] Karasawa K (2006) Clinical aspects of plasma platelet-activating factor-acetylhydrolase. *Biochim Biophys Acta* **1761**, 1359-1372.

[12] Kuwano R, Miyashita A, Arai H Asada T, Imagawa M, Shoji M, Higuchi S, Urakami K, Kakita A, Takahashi H, Tsukie T, Toyabe S, Akazawa K, Kanazawa I, Ihara Y (2006) Dynamin-binding protein gene on chromosome 10q is associated with late-onset Alzheimer's disease. *Hum Mol Genet* **15**, 2170-2182.

[13] Miyashita A, Arai H, Asada T, Imagawa M, Matsubara E, Shoji M, Higuchi S, Urakami K, Kakita A, Takahashi H, Toyabe S, Akazawa K, Kanazawa I, Ihara Y, Kuwano R (2007) Genetic association of CTNNA3 with late-onset Alzheimer's disease in females. *Hum Mol Genet* **16**, 2854-2869.

[14] Miyashita A, Arai H, Asada T, Imagawa M, Shoji M, Higuchi S, Urakami K, Toyabe S, Akazawa K, Kanazawa I, Ihara Y, Kuwano R (2009) GAB2 is not associated with late-onset Alzheimer's disease in Japanese. *Eur J Hum Genet* **17**, 682-686.

[15] Takei N, Miyashita A, Tsukie T, Arai H, Asada T, Imagawa M, Shoji M, Higuchi S, Urakami K, Kimura H, Kakita A, Takahashi H, Tsuji S, Kanazawa I, Ihara Y, Odani S, Kuwano R (2009) Genetic association study on in and around the APOE in late-onset Alzheimer disease in Japanese. *Genomics* **93**, 441-448.

[16] McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM (1984) Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. *Neurology* **34**, 939-944.

[17] Folstein MF, Folstein SE, McHugh PR (1975) "Mini-mental state". A practical method for grading the cognitive state of patients for the clinician. *J Psychiatr Res* **12**, 189-198.

[18] Morris JC (1993) The Clinical Dementia Rating (CDR): current version and scoring rules. *Neurology* **43**, 2412-2414.

[19] Reisberg B (1988) Functional assessment staging (FAST). *Psychopharmacol Bull* **24**, 653-659.

[20] Stafforini DM (2009) Functional consequences of mutations and polymorphisms in the coding region of the PAF acetylhydrolase (PAF-AH) gene. *Pharmaceuticals* **2,** 94-117.

[21] Reiman EM, Webster JA, Myers AJ, Hardy J, Dunckley T, Zismann VL, Joshipura KD, Pearson JV, Hu-Lince D, Huentelman MJ (2007) GAB2 alleles modify Alzheimer's risk in APOE epsilon4 carriers. *Neuron* **54**, 713-720.

[22] Bertram L, Lange C, Mullin K, Parkinson M, Hsiao M, Hogan MF, Schjeide BM, Hooli B, Divito J, Ionita I, Jiang H, Laird N, Moscarillo T, Ohlsen KL, Elliott K, Wang X, Hu-Lince D, Ryder M, Murphy A, Wagner SL, Blacker D, Becker KD, Tanzi RE (2008) Genome-wide association analysis reveals putative Alzheimer's disease susceptibility loci in addition to APOE. *Am J Hum Genet* **83**, 623-632.

[23] Li H, Wetten S, Li L, St Jean PL, Upmanyu R, Surh L, Hosford D, Barnes MR, Briley JD, Borrie M, Coletta N, Delisle R, Dhalla D, Ehm MG, Feldman HH, Fornazzari L, Gauthier S, Goodgame N, Guzman D, Hammond S, Hollingworth P, Hsiung GY, Johnson J, Kelly DD, Keren R, Kertesz A, King KS, Lovestone S, Loy-English I, Matthews PM, Owen MJ, Plumpton M, Pryse-Phillips W, Prinjha RK, Richardson JC, Saunders A, Slater AJ, St George-Hyslop PH, Stinnett SW, Swartz JE, Taylor RL, Wherrett J, Williams J, Yarnall DP, Gibson RA, Irizarry MC, Middleton LT, Roses AD (2008) Candidate single-nucleotide polymorphisms from a genomewide association study of Alzheimer disease. *Arch Neurol* **65**, 45-53.

[24] Beecham GW, Martin ER, Li YJ, Slifer MA, Gilbert JR, Haines JL, Pericak-Vance MA (2009) Genome-wide association study implicates a chromosome 12 risk locus for late-onset Alzheimer disease. *Am J Hum Genet* **84**, 35-43.

[25] Carrasquillo MM, Zou F, Pankratz VS, Wilcox SL, Ma L, Walker LP, Younkin SG, Younkin CS, Younkin LH, Bisceglio GD, Ertekin-Taner N, Crook JE, Dickson DW, Petersen RC, Graff-Radford NR, Younkin SG (2009) Genetic variation in PCDH11X is associated with susceptibility to late-onset Alzheimer's disease. *Nat Genet* **41**, 192-198.

[26] Harold D, Abraham R, Hollingworth P, Sims R, Gerrish A, Hamshere ML, Pahwa JS, Moskvina V, Dowzell K, Williams A, Jones N, Thomas C, Stretton A, Morgan AR, Lovestone S, Powell J, Proitsi P, Lupton MK, Brayne C, Rubinsztein DC, Gill M, Lawlor B, Lynch A, Morgan K, Brown KS, Passmore PA, Craig D, McGuinness B, Todd S, Holmes C, Mann D, Smith AD, Love S, Kehoe PG, Hardy J, Mead S, Fox N, Rossor M, Collinge J, Maier W, Jessen F, Schürmann B, van den Bussche H, Heuser I, Kornhuber J, Wiltfang J, Dichgans M, Frölich L, Hampel H, Hüll M, Rujescu D, Goate AM, Kauwe JS, Cruchaga C, Nowotny P, Morris JC, Mayo K, Sleegers K, Bettens K, Engelborghs S, De Deyn PP, Van Broeckhoven C, Livingston G, Bass NJ, Gurling H, McQuillin A, Gwilliam R, Deloukas P, Al-Chalabi A, Shaw CE, Tsolaki M, Singleton AB, Guerreiro R, Mühleisen TW, Nöthen MM, Moebus S, Jöckel KH, Klopp N, Wichmann HE, Carrasquillo MM, Pankratz VS, Younkin SG, Holmans PA, O'Donovan M, Owen MJ, Williams J (2009) Genome-wide association study identifies variants at CLU and PICALM associated with Alzheimer's disease. *Nat Genet* **41**, 1088-1093.

[27] Schnabel R, Dupuis J, Larson MG, Lunetta KL, Robins SJ, Zhu Y, Rong J, Yin X, Stirnadel HA, Nelson JJ, Wilson PW, Keaney JF, Vasan RS, Benjamin EJ (2009) Clinical and genetic factors associated with lipoprotein-associated phospholipase A2 in the Framingham Heart Study. *Atherosclerosis* **204**, 601-607.

Lipoprotein-Associated Phospholipase A2 Is Associated with Risk of Dementia

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Objective: **High levels of the inflammatory marker lipoprotein-associated phospholipase A2 (Lp-PLA2) have been proposed to be a predictor of coronary heart disease and stroke. Because both inflammation and vascular disease are associated with dementia, the objective of the present study was to examine the association between Lp-PLA2 and the risk of dementia.** ***Methods:*** **Within the Rotterdam Study, a population-based prospective cohort study, we performed a case–cohort study. Of the 6,713 participants at risk for dementia, a random sample of 1,742 individuals was drawn. During follow-up (mean, 5.7 years), 302 incident dementia cases were identified. Cox proportional hazard models were used to estimate the association of Lp-PLA2 and dementia.** ***Results:*** **We found that subjects with higher levels of Lp-PLA2 had an increased risk of dementia. Compared with the first quartile of Lp-PLA2, age- and sex-adjusted hazard ratios (HRs; 95% confidence interval [CI]) for dementia for the second, third, and fourth quartiles were 1.19 (0.78–1.81), 1.15 (0.74–1.79), and 1.56 (1.03–2.37), respectively (p value for trend 0.04). Additional adjustment for cardiovascular and inflammatory factors did not change the estimates.** ***Interpretation:*** **This is the first study to our knowledge that shows that Lp-PLA2 is associated with the risk of dementia independent of cardiovascular and inflammatory factors and provides evidence for a potential role of Lp-PLA2 in identifying subjects at risk for dementia.**

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Lipoprotein-associated phospholipase A2 (Lp-PLA2, also known as platelet-activating factor acetylhydrolase) has been proposed as a predictor of coronary heart disease independent of traditional cardiovascular risk factors.[1–4] Lp-PLA2 circulates in the blood, associated with low-density lipoprotein (LDL) cholesterol.[5] It hydrolyzes oxidized phospholipids to generate lysophosphatidylcholine and oxidized fatty acids, which are believed to have proinflammatory properties.[6] Lp-PLA2 may be an inflammatory marker or directly promote atherogenesis.[7] Recently, our group reported the association of higher levels of Lp-PLA2 not only with coronary heart disease but also with stroke.[8] Because inflammation markers,[9,10] cardiovascular risk factors,[11,12] and cerebrovascular disease[13,14] all may play a role in dementia, we hypothesized a relation between Lp-PLA2 levels and the risk of dementia. We investigated the association of Lp-PLA2 and risk of dementia in the Rotterdam Study among people aged 55 years and older.

Subjects and Methods

Study Population

The Rotterdam Study is a population-based prospective cohort study that investigates the incidence and risk factors of cardiovascular, neurodegenerative, locomotor, and ophthalmological diseases in the elderly.[15] From 1990 to 1993, all 10,275 residents aged 55 years or older of Ommoord, a district of the city of Rotterdam, were invited to participate in an extensive home interview and two visits to the research center, and 7,983 (78%) of them agreed. The medical ethics committee of the Erasmus Medical Center approved the study, and written informed consent was obtained from all participants.

At the baseline clinical examination, blood samples were drawn from 7,050 subjects of whom 7,047 underwent screening for dementia. In these, prevalent dementia was diagnosed in 334, resulting in a cohort with blood samples taken and at risk for dementia of 6,713 subjects. Follow-up examinations were conducted in 1993 to 1994 and in 1997 to 1999. In addition, through linkage with records of general practitioners, the total cohort was continuously monitored for major disease outcome. This resulted in a virtually complete follow-up until January 1, 2000.

Study Design

For reasons of efficiency, a case–cohort design[16,17] was used. In this design, a random sample, or "subcohort," is drawn from the source population. Subjects from the source population who are not included in the subcohort yet develop the disease are selected as additional cases and added to the anal-

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yses. Baseline exposure is measured in the cases and controls included in the subcohort and in the additional cases. In the case–cohort analysis, only subjects from the random cohort contribute to follow-up time. A random subcohort of 1,742 subjects was drawn from our cohort at risk, of whom 77 developed dementia during follow-up (Fig). We added 225 dementia patients who developed dementia outside the subcohort. Of the total of 302 incident dementia patients, Alzheimer's disease (AD) was diagnosed in 222 subjects, including 22 patients with AD accompanied by cerebrovascular disease, 44 with vascular dementia, 14 patients with dementia in Parkinson's disease, and 22 patients with dementia due to other causes such as multisystem atrophy, frontotemporal dementia, and Lewy body dementia.

Measurement of Lp-PLA2 Activity

Plasma aliquots prepared from nonfasted blood samples were collected at baseline and stored at −80°C, and Lp-PLA2 activity was measured with a high-throughput radiometric activity assay as described. In brief, plasma samples were divided into aliquots, placed in 96-well microtiter plates, and mixed with a substrate solution consisting of 0.4μmol/L [^{3}H]-PAF (specific activity 21.5Ci/mmol; Perkin-Elmer Life Sciences, Oak Brook, IL) and 99.6μmol/L C16-PAF (Avanti Polar Lipids, Alabaster, AL) in assay buffer (100mmol/L HEPES, 150mmol/L NaCl, 5mmol/L EDTA, pH 7.4). The reactions were allowed to proceed at room temperature for 5 minutes before the phospholipid substrates were sequestered by an ice-cold fatty acid–free bovine serum albumin solution at a final concentration of 16.1mg/ml. The bovine serum albumin–lipid complexes then were precipitated with ice-cold trichloroacetic acid at a final concentration of 7.8% and a pellet was created by centrifugation at approximately 6,000*g* for 15 minutes at 4°C. Aliquots of the supernatant containing the reaction products were transferred to another microplate (Perkin-Elmer) and the radioactivity counted in a Topcount liquid scintillation counter (Perkin-Elmer Life Sciences, Oak Brook, IL) upon addition of Microscint-20 scintillation cocktail (Perkin-Elmer Life Sciences). Lp-PLA2 activity was expressed as nanomoles of PAF hydrolyzed per minute per 1ml of plasma samples. On the basis of split samples, the coefficient of variation was 8.4%. Specimens of cases were assessed in the same runs as the random sample from the subcohort.

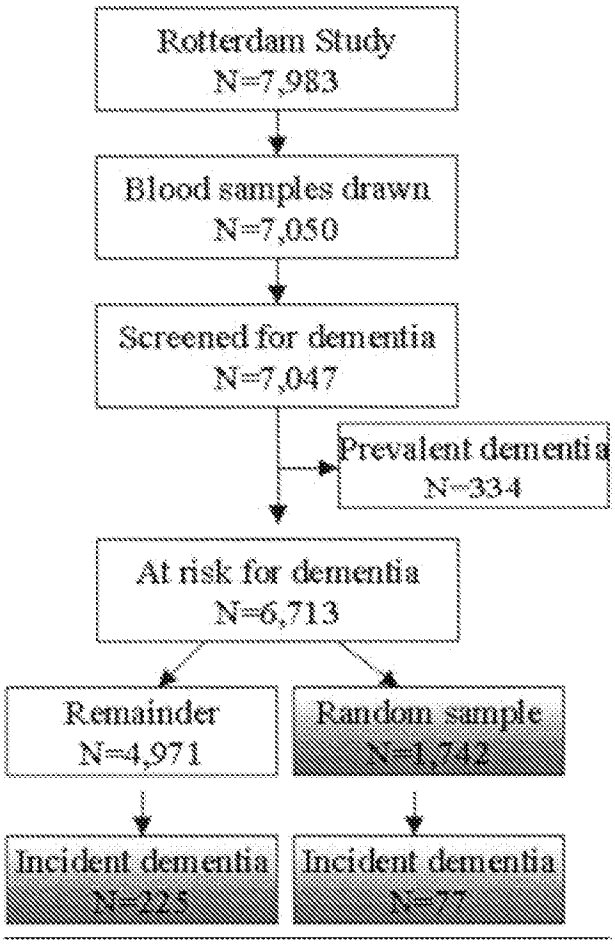

Fig. Description of study population. In the case–cohort analysis, the random sample, incident cases arising from the random sample and additional incident cases are included (shaded areas).

Covariates

At baseline, a trained investigator visited all participants at home and collected information using a computerized questionnaire. The interview included current health status, medical history, drug use, and smoking behavior. Additionally, during two visits to the research center, clinical measures were obtained. Height and weight were measured and the body mass index (BMI) was calculated (weight [kg]/height [m^2]). Blood pressure was measured at the right brachial artery using a random-zero sphygmomanometer with the participant in sitting position. Nonfasting blood samples were drawn and immediately frozen. Total cholesterol, high-density lipid (HDL) cholesterol and glucose were measured within 2 weeks, as described previously.[18] Immediately after blood sampling, white cell count was assessed in citrate plasma using a Coulter Counter T540 (Coulter Electronics, Luton, England), which has a coefficient of variation less than 2.0%. Quality of assessments was continuously monitored by Instruchemi (Hilversum, the Netherlands). Using a nephelometric method (Immage; Beckman Coulter Inc., Fullerton, CA), high sensitivity C-reactive protein was measured in blood samples that were kept frozen at −20°C. Genotyping for apolipoprotein E (APOE) was performed on coded DNA specimens without knowledge of diagnosis. A polymerase chain reaction was performed.[19] Two groups were formed on the basis of presence or absence of an APOE ε4 allele. Furthermore, ultrasonography of both carotid arteries was performed. We used presence of carotid plaques as an indicator of atherosclerosis. Carotid plaques were determined at six different locations in the carotid arteries: common carotid artery, carotid bifurcation, and internal carotid artery at both left and right side.[20] We defined diabetes mellitus as a random or postload glucose level greater than or equal to 11.1mmol/L or the use of blood glucose lowering medication.

Diagnosis of Dementia

During baseline and follow-up examinations the diagnosis of dementia followed a similar three-step protocol.[21] Two brief tests of cognition (Mini-Mental State Examination [MMSE][22] and Geriatric Mental State schedule [GMS]) organic level[23] were used to screen all subjects. Screen-positives (MMSE score <26 or GMS organic level >0) underwent further cognitive testing using the Cambridge Examination for Mental Disorders of the Elderly (CAMDEX).[24] Subjects who were suspected of having dementia were subsequently examined by a neurologist and a neuropsychologist and, if possible, had a magnetic resonance imaging scan. In addition, the total cohort was continuously monitored for incident dementia through computerized linkage between the study database and digitalized medical records from general practitioners and the Regional Institute for Outpatient Mental Health Care.[21] The diagnoses of dementia and Alzheimer's disease were made in accordance with internationally accepted criteria for dementia (Diagnostic and Statistical Manual of Mental Disorders III-R),[25] Alzheimer's disease (National Institute of Neurological and Communication Disorders Alzheimer's Disease and Related Disorders Association [NINCDS-ADRDA]),[26] and vascular dementia (National Institute of Neurological Disorders and Stroke [NINDS-Association Internationale pour la Recherche et l'Enseignement en Neurosciences (AIREN)])[27] by a panel including a neurologist, neuropsychologist, and research physician who reviewed all existing information.

Data Analysis

The association of Lp-PLA2 activity with dementia and subtypes of dementia was evaluated in a case–cohort design through Cox' proportional hazard models with modification of the standard errors based on robust variance estimates. We used the method according to Barlow in which the random cohort is weighted by the inverse of the sampling fraction from the source population.[16,17] Lp-PLA2 was normally distributed, and first we looked at the association of Lp-PLA2 and the risk of dementia and subtypes of dementia entering Lp-PLA2 as a linear term (per standard deviation [SD]) in the model. Then, quartiles of Lp-PLA2 were made and the lowest quartile was used as the reference category. Analyses were adjusted for age and sex, and additionally for the following confounders; BMI, total cholesterol, HDL cholesterol, systolic blood pressure, carotid plaques, current smoking, diabetes mellitus, white cell count, and C-reactive protein. Adjustment was made for presence of the APOE ε4 allele. In the test for trend analysis, we replaced quartiles of Lp-PLA2 activity by continuous values of Lp-PLA2 activity. To investigate whether the association between Lp-PLA2 and dementia was different for men and women, or varied with cholesterol levels, we looked at interactions of Lp-PLA2 and sex, and Lp-PLA2 and cholesterol. In analyses stratified by sex, we used sex-specific quartiles. To assess whether the association between Lp-PLA2 and dementia was mediated by stroke, we repeated the analyses excluding subjects with prevalent stroke and censoring those with incident stroke. To assess whether the association was influenced by lipid-lowering medication, in an additional analysis we excluded subjects who used lipid-lowering medication (statins or fibrates) at baseline. We had missing values for covariates in less than 5% (7.5% in C-reactive protein). We imputed the median value for missing values. Data analyses were performed using SAS 8.2 and SPSS 11.0 statistical software (SAS, Cary, NC).

Results

Characteristics of the random cohort are shown in Table 1. Increasing levels of Lp-PLA2 were associated with an increased risk of dementia (Table 2). People in the upper quartile had a 56% higher risk compared with those in the lower quartile. Additional adjustments for BMI, total cholesterol, HDL cholesterol, systolic blood pressure, carotid plaques, current smoking, diabetes mellitus, white cell count, and C-reactive protein did not markedly affect the estimates, nor did additional adjustment for presence of APOE ε4 allele. We looked at the associations in men and women separately (Table 3). The relation between Lp-PLA2 and risk of dementia seemed more pronounced in men. However, the p value of the interaction term between sex and Lp-PLA2 activity was 0.07.

We found no indication that the relation between Lp-PLA2 and dementia varied with cholesterol levels (p value interaction term between total cholesterol

Table 1. Characteristics of the Random Cohort (n = 1,742) and the Total Cohort at Risk (n = 6,713)

Variable	Total Cohort at Risk	Random Cohort
Age (yr) (SD)	69.5 (9.1)	68.5 (8.6)
Women (%)	59.9	61.0
Body mass index (kg/m^2) (SD)	26.3 (4.0)	26.2 (3.6)
Systolic blood pressure (mm Hg) (SD)	139.3 (22.3)	138.1 (22.2)
Diastolic blood pressure (mm Hg) (SD)	73.6 (11.5)	73.4 (11.1)
Total cholesterol (mmol/L) (SD)	6.6 (1.2)	6.7 (1.2)
High-density lipid cholesterol (mmol/L) (SD)	1.3 (0.4)	1.3 (0.4)
Diabetes (%)	10.0	9.5
Smokers (%)		
Current	22.8	23.1
Former	41.6	41.0
Lp-PLA2 activity (nmol/min/ml plasma) (SD)	—	44.5 (11.5)

SD = standard deviation.

Table 2. Hazard Ratio (95% confidence interval) for Dementia

	Model 1[a]	Model 2[b]
Lp-PLA2 per SD increase	1.12 (0.97–1.28)	1.16 (0.97–1.37)
First quartile	1.00 (reference)	1.00 (reference)
Second quartile	1.19 (0.78–1.81)	1.24 (0.80–1.92)
Third quartile	1.15 (0.74–1.79)	1.23 (0.76–1.98)
Fourth quartile	1.56 (1.03–2.37)	1.74 (1.07–2.83)
p trend	0.04	0.03

[a]Hazard ratio (95% confidence interval) adjusted for age and sex.
[b]Hazard ratio (95% confidence interval) adjusted for body mass index, total cholesterol, high-density lipid cholesterol, systolic blood pressure, carotid plaques, current smoking, diabetes mellitus, white cell count, and C-reactive protein.

SD = standard deviation.

and Lp-PLA2 activity 0.14). The *p* value of the interaction term between HDL cholesterol and Lp-PLA2 was 0.77. No differences between men and women were found.

When we investigated subtypes of dementia, the association of Lp-PLA2 appeared stronger with vascular dementia than with AD (Table 4). No clear sex effect was found for these subtypes of dementia.

Because an association of Lp-PLA2 with stroke has been reported, and stroke is associated with an increased risk of dementia, we subsequently excluded all people who had experienced a stroke before entering our study (n = 53) and the people with missing information regarding previous stroke (n = 35) and censored the incident stroke cases (n = 103). This only marginally changed the effect estimates; if anything, the effect became stronger. Compared with the first quartile, age- and sex-adjusted HRs of the second, third, and fourth quartile were 1.16 (95% CI, 0.74–1.82), 1.15 (95% CI, 0.71–1.87), and 1.64 (95% CI, 1.06–2.54), *p* value for trend 0.03. After additional adjustment these estimates were 1.20 (95% CI, 0.75–1.91), 1.23 (95% CI, 0.74–2.05), and 1.80 (95% CI, 1.08–2.99), *p* value for trend 0.03. Excluding subjects who used lipid-lowering medication at baseline (n = 40) did not change the estimates.

Discussion

We found that higher plasma levels of Lp-PLA2 activity were associated with an increased risk of dementia. In particular, subjects in the highest quartile of Lp-PLA2 levels were at higher risk of dementia. The association was independent of C-reactive protein and cardiovascular risk factors and could not be explained by previous or incident stroke.

The strengths of our study are its prospective design and the population-based setting with a large number of subjects. Furthermore, the follow-up with respect to the diagnosis of dementia was virtually complete and selection bias is unlikely. Although we adjusted for many potential confounders, residual confounding could have occurred. Lp-PLA2 was measured in nonfasting blood, which may have increased variability in levels and thereby may have reduced the power in our study to find an association. Also, because no data were available on LDL cholesterol, we could only adjust for total cholesterol and HDL cholesterol in our analyses.

Lp-PLA2 is a marker of systemic inflammation. Levels in animals are substantially increased after injecting endotoxin and plasma levels are increased in a variety of inflammatory conditions.[5,28] Furthermore, Lp-PLA2 is produced mainly by macrophages and expression is regulated by inflammatory cytokines.[29]

To investigate whether the association between Lp-PLA2 and the risk of dementia could be explained through an inflammatory pathway, we adjusted for C-reactive protein and white cell count in our multivariate analyses. However, this did not change the estimate. This is in line with observations on cardiovascular disease and stroke, where Lp-PLA2 was found to be a risk factor independent of C-reactive protein and white cell count.[2,8] Moreover, we found no correlation between levels of C-reactive protein and levels of Lp-

Table 3. Hazard Ratio (95% confidence interval) for Dementia by Sex

	Women		Men	
	Model 1[a]	Model 2[b]	Model 1[a]	Model 2[b]
Lp-PLA2 per SD increase	1.01 (0.85–1.21)	1.06 (0.84–1.33)	1.29 (1.03–1.63)	1.22 (0.93–1.60)
First quartile	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)
Second quartile	1.17 (0.69–1.99)	1.28 (0.74–2.24)	0.90 (0.43–1.88)	0.90 (0.39–2.06)
Third quartile	1.20 (0.70–2.07)	1.42 (0.77–2.61)	1.55 (0.77–3.13)	1.35 (0.64–2.83)
Fourth quartile	1.21 (0.71–2.06)	1.50 (0.80–2.82)	1.77 (0.92–3.14)	1.51 (0.70–3.25)
p trend	0.53	0.23	0.04	0.19

[a]Hazard ratio (95% confidence interval) adjusted for age and sex.
[b]Hazard ratio (95% confidence interval) adjusted for body mass index, total cholesterol, high-density lipid cholesterol, systolic blood pressure, carotid plaques, current smoking, diabetes mellitus, white cell count, and C-reactive protein.

SD = standard deviation.

Table 4. Hazard Ratio (95% confidence interval) by Dementia Subtype

	Alzheimer's disease		Vascular Dementia	
	Model 1[a]	Model 2[b]	Model 1[a]	Model 2[b]
Lp-PLA2 per SD increase	1.06 (0.91–1.24)	1.09 (0.90–1.32)	1.20 (0.92–1.57)	1.18 (0.88–1.82)
First quartile	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)
Second quartile	1.12 (0.71–1.77)	1.16 (0.72–1.84)	0.82 (0.24–2.84)	0.78 (0.22–2.73)
Third quartile	1.00 (0.61–1.62)	1.03 (0.61–1.74)	1.48 (0.49–4.50)	1.38 (0.38–5.01)
Fourth quartile	1.30 (0.82–2.04)	1.38 (0.82–2.34)	2.19 (0.80–6.03)	2.02 (0.59–6.88)
p trend	0.35	0.29	0.06	0.16

[a]Hazard ratio (95% confidence interval) adjusted for age and sex.
[b]Hazard ratio (95% confidence interval) adjusted for body mass index, total cholesterol, high-density lipid cholesterol, systolic blood pressure, carotid plaques, current smoking, diabetes mellitus, white cell count, and C-reactive protein.

SD = standard deviation.

PLA2, in accordance with results of previous studies.[2] A possible explanation for this is that C-reactive protein and Lp-PLA2 are produced in different tissues in response to different patterns of cytokines.[7] We found only a weak correlation between levels of Lp-PLA2 and white cell count.

Note that there was not a compelling gradient of risk of dementia for levels of Lp-PLA2. Although we found a significant trend over the quartiles, in particular, subjects in the highest quartile of Lp-PLA2 were at higher risk of dementia. Possibly, a threshold effect is present, although this has not been found in relation to stroke and coronary heart disease. Also, the association between Lp-PLA2 and risk of dementia (hazard ratio [HR] for the upper quartile compared with the lower 1.56 [95% CI, 1.03–2.37]) was weaker than between Lp-PLA2 and ischemic stroke and coronary heart disease (HR for the upper quartile compared with the lower 1.97 [95% CI, 1.04–3.74] and 2.36 [95% CI, 1.58–3.52], respectively)[8] in the Rotterdam Study. It is important that our findings are replicated.

There is strong evidence that inflammation is important in dementia. Elevated levels of inflammatory proteins such as α-1-antichymotrypsin and IL-6 were found in plasma of patients with Alzheimer's disease years before the clinical dementia syndrome developed.[9] Furthermore, a beneficial effect of nonsteroidal antiinflammatory drugs has been suggested.[30–33] How exactly peripheral inflammatory markers reflect or affect processes in the brain is not known. The notion that atherosclerosis plays a role in this is supported by our observation that the association of high levels of Lp-PLA2 seemed stronger in vascular dementia than in Alzheimer's disease. However, the hypothesis of a vascular mechanism is not supported by the finding that adjusting for atherosclerosis measures did not change the estimates. Because we could take into account only clinical stroke, we cannot rule out the possibility that the relation is mediated by subclinical vascular events such as silent brain infarcts, which are important risk factors for dementia.[14]

An alternative explanation would be that Lp-PLA2 levels could directly affect the brain or key molecules that are implicated in Alzheimer's disease such as amyloid and tau. However, we are not aware of such a relation.

The role of cholesterol is not clear. In plasma, 80% of Lp-PLA2 circulates bound to LDL cholesterol and HDL cholesterol contains 15 to 20% of the enzyme mass or activity. The Atherosclerosis Risk in Communities study found an independent effect of Lp-PLA2 on cardiovascular disease in subjects with LDL cholesterol below the median only.[3] The same relation was found in the West of Scotland Coronary Prevention Study Group, a study conducted among men with elevated cholesterol levels.[2] In the Rotterdam Study, in men and women, the association with cardiovascular disease was present over the entire range of non–HDL cholesterol. Looking at dementia, we did not find significant interactions of Lp-PLA2 and total cholesterol or HDL cholesterol. After adjustment for total cholesterol and HDL cholesterol, the association between Lp-PLA2 and dementia in women became slightly stronger, whereas in men the association attenuated. We have no specific explanation for these differences. Note that the role of cholesterol in dementia is far less clear than it is in cardiovascular disease. Moreover, in our data cholesterol was not associated with the risk of dementia.

Our data suggest that the relation between Lp-PLA2 and the risk of dementia may be different in men and women. It is not uncommon for a risk factor to have sex-specific effects.[34] In our study, the interaction term of sex and mean Lp-PLA2 activity was only borderline significant, although this might also be caused by a lack of power. Regarding cardiovascular disease, the Women's Health Study did not find an association with Lp-PLA2 in women after adjustment for cardiovascular risk factors.[35] The importance of Lp-PLA2 is increasingly being recognized in cardiovascular disease, and measuring plasma Lp-PLA2 levels in the clinical setting to predict the risk of coronary heart disease, together with the more standard predictors such as cho-

lesterol, is now being considered. Our data suggest that it is also worthwhile to further investigate Lp-PLA2 as a possible novel risk factor for dementia.

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References

1. Caslake MJ, Packard CJ, Suckling KE, et al. Lipoprotein-associated phospholipase A(2), platelet-activating factor acetylhydrolase: a potential new risk factor for coronary artery disease. Atherosclerosis 2000;150:413–419.
2. Packard CJ, O'Reilly DS, Caslake MJ, et al. Lipoprotein-associated phospholipase A2 as an independent predictor of coronary heart disease. West of Scotland Coronary Prevention Study Group. N Engl J Med 2000;343:1148–1155.
3. Ballantyne CM, Hoogeveen RC, Bang H, et al. Lipoprotein-associated phospholipase A2, high-sensitivity C-reactive protein, and risk for incident coronary heart disease in middle-aged men and women in the Atherosclerosis Risk in Communities (ARIC) study. Circulation 2004;109:837–842.
4. Caslake MJ, Packard CJ. Lipoprotein-associated phospholipase A2 (platelet-activating factor acetylhydrolase) and cardiovascular disease. Curr Opin Lipidol 2003;14:347–352.
5. Stafforini DM, McIntyre TM, Zimmerman GA, Prescott SM. Platelet-activating factor acetylhydrolases. J Biol Chem 1997; 272:17895–17898.
6. MacPhee CH, Moores KE, Boyd HF, et al. Lipoprotein-associated phospholipase A2, platelet-activating factor acetylhydrolase, generates two bioactive products during the oxidation of low-density lipoprotein: use of a novel inhibitor. Biochem J 1999;338:479–487.
7. Rader DJ. Inflammatory markers of coronary risk. N Engl J Med 2000;343:1179–1182.
8. Oei HH, van der Meer IM, Hofman A, et al. Lipoprotein-associated phospholipase A2 activity is associated with risk of coronary heart disease and ischemic stroke: the Rotterdam Study. Circulation 2005;111:570–575.
9. Engelhart MJ, Geerlings MI, Meijer J, et al. Inflammatory proteins in plasma and the risk of dementia: the Rotterdam Study. Arch Neurol 2004;61:668–672.
10. Schmidt R, Schmidt H, Curb JD, et al. Early inflammation and dementia: a 25-year follow-up of the Honolulu-Asia Aging Study. Ann Neurol 2002;52:168–174.
11. de la Torre JC. Alzheimer disease as a vascular disorder: nosological evidence. Stroke 2002;33:1152–1162.
12. Pansari K, Gupta A, Thomas P. Alzheimer's disease and vascular factors: facts and theories. Int J Clin Pract 2002;56: 197–203.
13. Honig LS, Tang MX, Albert S, et al. Stroke and the risk of Alzheimer disease. Arch Neurol 2003;60:1707–1712.
14. Vermeer SE, Prins ND, den Heijer T, et al. Silent brain infarcts and the risk of dementia and cognitive decline. N Engl J Med 2003;348:1215–1222.
15. Hofman A, Grobbee DE, de Jong PT, van den Ouweland FA. Determinants of disease and disability in the elderly: the Rotterdam Elderly Study. Eur J Epidemiol 1991;7:403–422.
16. Barlow WE. Robust variance estimation for the case-cohort design. Biometrics 1994;50:1064–1072.
17. Barlow WE, Ichikawa L, Rosner D, Izumi S. Analysis of case-cohort designs. J Clin Epidemiol 1999;52:1165–1172.
18. Meijer WT, Hoes AW, Rutgers D, et al. Peripheral arterial disease in the elderly: the Rotterdam Study. Arterioscler Thromb Vasc Biol 1998;18:185–192.
19. Slooter AJ, Cruts M, Kalmijn S, et al. Risk estimates of dementia by apolipoprotein E genotypes from a population-based incidence study: the Rotterdam Study. Arch Neurol 1998;55:964–968.
20. Bots ML, Hoes AW, Koudstaal PJ, et al. Common carotid intima-media thickness and risk of stroke and myocardial infarction: the Rotterdam Study. Circulation 1997;96: 1432–1437.
21. Ott A, Breteler MM, van Harskamp F, et al. Incidence and risk of dementia. The Rotterdam Study. Am J Epidemiol 1998;147: 574–580.
22. Folstein MF, Folstein SE, McHugh PR. "Mini-mental state." A practical method for grading the cognitive state of patients for the clinician. J Psychiatr Res 1975;12:189–198.
23. Copeland JR, Kelleher MJ, Kellett JM, et al. A semi-structured clinical interview for the assessment of diagnosis and mental state in the elderly: the Geriatric Mental State Schedule. I. Development and reliability. Psychol Med 1976;6:439–449.
24. Roth M, Tym E, Mountjoy CQ, et al. CAMDEX. A standardised instrument for the diagnosis of mental disorder in the elderly with special reference to the early detection of dementia. Br J Psychiatry 1986;149:698–709.
25. American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders. Washington, DC: American Psychiatric Association, 1987.
26. McKhann G, Drachman D, Folstein M, et al. Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. Neurology 1984;34:939–944.
27. Roman GC, Tatemichi TK, Erkinjuntti T, et al. Vascular dementia: diagnostic criteria for research studies. Report of the NINDS-AIREN International Workshop. Neurology 1993;43: 250–260.
28. Memon RA, Fuller J, Moser AH, et al. In vivo regulation of plasma platelet-activating factor acetylhydrolase during the acute phase response. Am J Physiol 1999;277:R94–R103.
29. Cao Y, Stafforini DM, Zimmerman GA, et al. Expression of plasma platelet-activating factor acetylhydrolase is transcriptionally regulated by mediators of inflammation. J Biol Chem 1998;273:4012–4020.
30. in t' Veld BA, Ruitenberg A, Hofman A, et al. Nonsteroidal antiinflammatory drugs and the risk of Alzheimer's disease. N Engl J Med 2001;345:1515–1521.
31. Rogers J, Kirby LC, Hempelman SR, et al. Clinical trial of indomethacin in Alzheimer's disease. Neurology 1993;43: 1609–1611.
32. Breitner JC, Welsh KA, Helms MJ, et al. Delayed onset of Alzheimer's disease with nonsteroidal anti-inflammatory and histamine H2 blocking drugs. Neurobiol Aging 1995;16:523–530.
33. McGeer PL, Schulzer M, McGeer EG. Arthritis and anti-inflammatory agents as possible protective factors for Alzheimer's disease: a review of 17 epidemiologic studies. Neurology 1996;47:425–432.
34. Ariyo AA, Thach C, Tracy R. Lp(a) lipoprotein, vascular disease, and mortality in the elderly. N Engl J Med 2003;349: 2108–2115.
35. Blake GJ, Dada N, Fox JC, et al. A prospective evaluation of lipoprotein-associated phospholipase A(2) levels and the risk of future cardiovascular events in women. J Am Coll Cardiol 2001;38:1302–1306.

Cancer Treatment

Mark Levin, MD; April 2008

Lipoprotein-associated phospholipase A2

Lipoprotein-associated phospholipase A2 (Lp-PLA2) is an enzyme that is produced by inflammatory cells, co-travels with circulating low-density lipoprotein (LDL), and hydrolyzes oxidized phospholipids in LDL. Its biological role has been controversial with initial reports purporting atheroprotective effects of Lp-PLA2 thought to be a consequence of degrading platelet-activating factor and removing polar phospholipids in modified LDL. Recent studies, however, focused on pro-inflammatory role of Lp-PLA2 mediated by products of the Lp-PLA2 reaction (lysophosphatidylcholine and oxidized nonesterified fatty acids). These bioactive lipid mediators, which are generated in lesion-prone vasculature and to a lesser extent in the circulation (eg, in electronegative LDL), are known to elicit several inflammatory responses. The proinflammatory action of Lp-PLA2 is also supported by a number of epidemiology studies suggesting that the circulating level of the enzyme is an independent predictor of cardiovascular events, despite some attenuation of the effect by inclusion of LDL, the primary carrier of Lp-PLA2, in the analysis.

Ascribing a role for Lp-PLA2, an enzyme that is produced by inflammatory cells, is transported on circulating LDL and hydrolyzes oxidized phospholipids in LDL, has been controversial. A growing number of epidemiological studies suggest that Lp-PLA2 is an independent predictor of cardiovascular events, despite some attenuation of this relationship by LDL, the primary carrier of Lp-PLA2. These observations strengthen the rationale to explore causal links between Lp-PLA2 and plaque vulnerability. The connection to cardiovascular diseae and Alzheimer's remain unproven; neither are the effects of interventions to reduce the level of this marker known. Empire considers this test to be be not medically necessary.

A.-L. Levonen, E. Vahakangas, J. K. Koponen, and S. Yla-Herttuala
Antioxidant Gene Therapy for Cardiovascular Disease: Current Status and Future Perspectives
Circulation, April 22, 2008; 117(16): 2142 - 2150.

W. Koenig
Cardiovascular Biomarkers: Added Value With an Integrated Approach?
Circulation, July 3, 2007; 116(1): 3 - 5.

A. Oldgren, S. K. James, A. Siegbahn, and L. Wallentin
Lipoprotein-associated phospholipase A2 does not predict mortality or new ischaemic events in acute coronary syndrome patients
Eur. Heart J., March 2, 2007; 28(6): 699 - 704.

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